REMARKS

Claims 1-3, 5-11 and 15-17 are pending. Claim10 is amended to independent form. No new matter is introduced by the amendment.

All claims remain rejected under 35 U.S.C. § 103(a). Applicants respectfully traverse the rejection of the aforementioned claims for the reasons set forth below.

I. The Claims are Not Obvious under 35 U.S.C. § 103(a)

The Examiner has maintained the rejections of Claims 1-3, 5-11 and 15-17 under 35 U.S.C. § 103(a) as being rendered obvious by Max *et al.* (Int. J. Cancer 1997;71:320-324; "Max") in view of Taylor *et al.* (Taylor *et al.*, Blood 1997;89:4078-4084; "Taylor"), Möhle *et al.* (Proc. Natl. Acad. Sci. USA 1997;94:663-668; "Möhle") and Charo *et al.* (J. Biol. Chem. 1987;262:9935-9938; "Charo") as evidenced by Coller *et al.* (Haemostasis 1996;26:285-293; "Coller").

The Examiner has not been persuaded by Applicants' arguments distinguishing the claimed invention from the cited references, or their combination. First, the Examiner has not agreed with Applicants' assessment of Max, which, briefly, is that the data presented in Max, which shows expression of $\alpha_v\beta_3$ on both malignant and non-malignant cells (albeit at lower levels on the latter) undermine the use of Max to render the claimed invention obvious. Second, the Examiner has questioned Applicants' mention of Brooks as a reference, where Applicants have taken the position that Max does not provide incremental teaching over Brooks, which is no longer being cited against the claims. Third, the Examiner has not accepted Applicants' position that the citations relating to GpIIb/IIIa, Taylor, Möhle and Charo, are not properly combined with Max.

Accordingly, the Examiner continues to contend as follows:

(1) Max teaches expression of $\alpha_v\beta_3$ on tumor cells and cells involved in angiogenesis; that angiogenesis contributes to diabetic retinopathy; that an antibody to $\alpha_v\beta_3$ detected integrin in cells; and that tumors can be treated with $\alpha_v\beta_3$ antagonists;

(2) Taylor teaches the use of 7E3 and related molecules, in the claimed dosages and routes of administration, for protection of baboons against microangiopathic hemolytic anemia and microvascular thrombotic renal failure;

(3) Charo teaches that administration of 7E3 inhibits platelet aggregation by binding to GPIIb/IIIa ($\alpha_{IIb}\beta_3$); and

(4) Möhle teaches that VEGF is produced from activated platelets, that VEGF is delivered to the site of injury by activated platelets, and that local secretion of VEGF may initiate angiogenesis; so that

(5) "it would have been obvious to use an antagonist of both $\alpha_v\beta_3$ and GPIIb/IIIa and use the 7E3 antibody which obviously have the claimed properties of the antagonist."

Applicants request the Examiner to reconsider the basis for the rejection and to afford Applicants the opportunity to restate, and hopefully clarify, their position regarding Max.

As background, Max states (at page 320, paragraph bridging first and second columns):

> Recent findings in a severe combined immunodeficiency (SCID) mouse/human skin chimeric model indicated that the intravenous application of a function-blocking monoclonal antibody (MAb) (LM609) directed to the $\alpha_v\beta_3$-complex is able to reduce the growth and invasiveness of human tumors. This seems to be due to the perturbation of angiogenesis induced by the tumors, with no apparent effect on surrounding normal tissue (Brooks et al., 1995). . . These data suggest that integrin $\alpha_v\beta_3$ might be a useful therapeutic target for cancer and other diseases characterized by pathological angiogenesis.

Max then, in the next paragraph, goes on to state (*emphasis added*):

> Several studies have investigated the distribution of α_v - and β_3- integrins on vessels (Mechtersheimer et al., 1994; Luscinskas et al., 1994) but *until now the use of a complex-specific antibody against the most interesting target, $\alpha_v\beta_3$, has been limited.* As different α_v - andβ_3-integrins are known, the reagents directed to individual chains must give ambiguous results.

Applicants interpret these statements to mean that while LM609 appeared to inhibit angiogenesis, previous attempts to evaluate the distribution of the individual integrins were inadequate to address whether the distribution of $\alpha_v\beta_3$ on cells would support the relevance of this complex to LM609's purported anti-angiogenic effect. Max then went on to use LM609 antibody to show the expression pattern of the $\alpha_v\beta_3$ *complex*, and concluded (at page 323, last paragraph, *emphasis added*):

> In summary, our data confirm and extend earlier reports on the upregulation of $\alpha_v\beta_3$ expression on the tumor vasculature. *However, they also show that $\alpha_v\beta_3$ expression is not limited to areas of neovascularization and that the expression in normal tissue does not appear to be due to hypoxia or to a tissue sampling artifact*;

and (at page 324, second paragraph)

> There are as yet no clues regarding the function of $\alpha_v\beta_3$ expression in normal tissue.

Therefore, a fair reading of Max would be that while the up-regulation of $\alpha_v\beta_3$ in tumor vasculature is *consistent* with a functional role for $\alpha_v\beta_3$ in tumor angiogenesis, the presence of $\alpha_v\beta_3$ on normal cells is not. Applicants' previous arguments were intended to highlight this ambiguity. Applicants position is, that the ambiguity regarding the role of $\alpha_v\beta_3$ would mean that it would not have been obvious, at the time the application was filed, that an antibody that is an antagonist of integrins $\alpha_v\beta_3$ and GpIIb/IIIa, such as 7E3, would inhibit angiogenesis.

To date, the Examiner has cited a number of references, including Max, that suggest an association between $\alpha_v\beta_3$ and angiogenesis. To support their arguments regarding the ambiguity of this association, Applicants have looked to the art, and found the following references, which they wish to bring to the Examiner's attention. All derive from the laboratory of Dr. Richard Hynes at the Massachusetts Institute of Technology.

First, Applicants invite the Examiner's attention to Abstract No. 1951, Taverna et al., March 1997, Proc. Am. Assoc. Cancer Res. 38:290-291 ("Taverna 1," attached hereto as EXHIBIT A), entitled "Is α5 Integrin A Tumor Suppressor Gene", which reports conflicting data observed regarding whether or not α_v has an anti-tumor effect.

Second, Applicants invite the Examiner's attention to Hynes and Bader, 1997, Thrombosis and Haemostasis 78(1):83-87 ("Hynes and Bader," attached hereto as EXHIBIT B) which reviews genetic analysis of integrins , and which states (at page 85, first column, first full paragraph):

> α_v integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) have been implicated in vasculogenesis and angiogenesis (21). For these reasons, we ablated the α_v gene (Bader and Hynes, unpublished data). Surprisingly, there was extensive development of the heart and vasculature. Around 20% of α_v -null progeny develop to term, are born alive but die soon afterward. These pups show hemorrhage in the brain and intestine . . .One interpretation would be that cardiogenesis and vasculogenesis is α_v -independent but that development and/or maintenance of the brain vasculature does depend on α_v integrins. It is possible that different forms of angiogenesis exist, which differ in their requirements for α_v integrins. It is interesting to note that knockouts of β_3, β_5 and β_6 are all viable with no obvious defects in vascular development . . .Overall, the effects of mutations of α_v integrins are all milder than might have been anticipated.

Third, Applicants would invite the Examiner's attention to a current publication, Taverna et al., 2004, Proc. Natl. Acad. Sci. U.S.A. 101:763-768 ("Taverna 2," attached hereto as EXHIBIT C) which is entitled "Increased primary tumor growth in mice null for β_3 - or β_3/β_5

integrins or selectins," which states (at page 767, second paragraph of "Discussion"):

> Prior work has suggested that α_v -integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) could act as negative regulators of angiogenesis [citations], and, indeed, we see enhanced vascularization of transplanted human tumors in mice lacking these integrins.

Taverna 1 and Hynes and Bader shortly precede the priority date of the instant application. Taverna 1 reports uncertainty as to whether α_v has tumor suppressive effects and Hynes and Bader teach at least ambiguous roles for both α_v and β_3 in angiogenesis. These references indicate that at the time the present application was filed, based on the state of the art, the skilled artisan would *not* have had reason to expect that the claimed antagonism of $\alpha_v\beta_3$ and GpIIb/IIIa would be anti-angiogenic. The most recent of the three references cited, Taverna 2, would teach away from the use of an $\alpha_v\beta_3$ antagonist as an anti-angiogenic agent.

Therefore, Applicants assert that in view of the ambiguity of the data in Max, which showed $\alpha_v\beta_3$ expressed on normal cells (albeit at a lower level than malignant cells) and the ambiguous observations of references such as Taverna 1 and Hynes and Bader, the skilled artisan would not have expected an antagonist of $\alpha_v\beta_3$ and GpIIb/IIIa to inhibit angiogenesis, even if Taylor, Charo and Möhle were, for the sake of argument, combined to teach that activated platelets may secrete VEGF and that 7E3 binds, via GpIIb/IIIa, to activated platelets.

Moreover, Applicants respectfully remind the Examiner of the data reported in Trikha et al., 2002, Cancer Research 62:2824-2833 ("Trikha," attached for the Examiner's convenience as EXHIBIT D), made of record in Applicants' response filed July 7, 2003. Trikha reports that in their experiments, in SCID mice, m7E3F(ab')$_2$ only partially inhibited the growth of human melanoma tumors. "Because c7E3 and m7E3F(ab')$_2$ do not cross-react with murine integrins, this inhibition of metastasis and tumor growth is attributable to direct blockade of

human $\alpha_v\beta_3$ integrins."1 In contrast, in nude rats, "where m7E3F(ab')$_2$ simultaneously binds to both human tumor and host platelet GPIIb/IIIa[$\alpha_{IIb}\beta_3$] and endothelial $\alpha_v\beta_3$ integrins" m7E3F(ab')$_2$ "completely blocked human tumor formation and growth of human melanoma tumors."2 Trikha 2 concludes that:

> In this rat xenograft model, which mimics the clinical situation, combined antiangiogenic and antitumor activity of m7E3F(ab')$_2$ was superior at inhibiting tumor growth when compared with its antitumor activity in the mouse xenograft model.

Figure 9 of Trikha at page 2831 shows the inhibition of angiogenesis into Matrigel plugs in nude rats by m73F(ab)'$_2$.

Trikha demonstrates the success of the claimed invention. Such results would not have been expected based on any of Max, Taylor, Charo, Möhle, Coller, or any combination thereof.

Accordingly, the claimed invention is not obvious over the cited references and it is respectfully requested that the instant rejection be withdrawn.

In addition, Applicants invite the Examiner's attention to Lincoff et al., 2001, "Abciximab Suppresses the Rise in Levels of Circulating Inflammatory Markers After Percutaneous Coronary Revascularization," Circulation 104:163-167 ("Lincoff et al.,"see also comments by Ray and Response by Lincoff et al., 2002, Circulation 105:e74, attached hereto as EXHIBIT E). Lincoff et al. identifies "abciximab" as c7E3 and supports claims in which 7E3 inhibits the inflammatory process, as recited in amended claims 10 and 15. Please see page 166 of Lincoff, which states (beginning in the last paragraph of column 1):

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1 Trikha 2, Abstract.
2 *Id.*

> Mortality reduction has not been observed to date with eptifibatide or tirofiban. Apparent heterogeneity in the magnitude of treatment effect observed in these trials between the antibody fragment and the reversible small molecule inhibitors may reflect differences in the intensity and duration of receptor blockade, inadequate dosing, variations in trial design, or statistical chance. *However, differences in receptor specificity among the agents may also be important. Eptifibatide and tirofiban inhibit only GP IIb/IIIa, but abciximab also binds to the* $\alpha_v\beta_3$ *(vitronectin) receptor on endothelial, smooth muscle, and inflammatory cells* and to an activated conformation of αMβ2 receptor on leukocytes (*emphasis added).*

Applicants further invite the Examiner's attention to a more recent article, Kereiakes, 2003, "Inflammation as a therapeutic target: a unique role for abciximab," Am. Heart J. 146(4 Suppl):S1-S4 (attached hereto as EXHIBIT F), which states, at p. S2:

> *A potent direct and sustained anti-inflammatory effect of abciximab after PCI has been demonstrated.* The durability of abciximab's anti-inflammatory effects may, in part, be due to the pharmacodynamic profile of this agent, which exhibits "redistribution" across cellular receptors. Indeed, as reflected by GP IIB/IIIa receptor occupancy, abciximab lingers in the circulation for > 2 weeks alter bolus administration. *Abciximab also binds the vitronectin* ($\alpha_v\beta_3$) *receptor with the same affinity as the glycoprotein (GP) IIb/IIIa receptor. Inhibition of the vitronectin receptor inhibits leukocyte adherence and leukocyte transmigration across endothelial cells (emphasis added).*

It is earnestly requested that the foregoing references be considered as supporting evidence for the patentability of claims 10, 11 and 15-17.

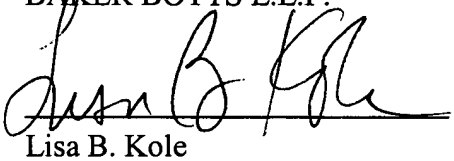
CONCLUSION

Based on the foregoing remarks and in light of the amendments, Applicants submit that the present application is in condition for allowance. A Notice of Allowance is therefore respectfully requested.

If a telephone interview would be of assistance in advancing the prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

Respectfully submitted,

BAKER BOTTS L.L.P.



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tissue, CD44 aberrations could serve as potential markers for adenocarcinoma of the ovary and provide insights into cancer-specific deregulation of mRNA processing.

#1945 Endothelial CD44 is involved in tumor angiogenesis. Griffioen, A.W., Coenen, M.J.H., Damen, C.A., Hellwig, S.M.M., Blijham, G.H., and Groenewegen, G. *Dept. of Internal Medicine and Medical Oncology, Laboratory for Angiogenesis Research, University Hospital Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

CD44 is an activation molecule in a number of different cell types. We investigated the expression of CD44 on human endothelial cells (EC) in relation to tumor angiogenesis. Using flow cytometry we showed that EC from the vasculature of human solid tumors display an enhanced expression of CD44 as compared to EC from normal tissue. This finding was confirmed by immunohistochemical studies on frozen tissue sections. Since tumors are dependent on angiogenesis, the role of angiogenic stimuli in the enhanced CD44 expression was investigated. We found that basic fibroblast growth factor (bFGF) and vascular endothelial growth factor were able to efficiently upregulate CD44 expression on cultured human EC. The upregulation reached maximal levels after treatment for 3 days with 10 ng/ml bFGF. The functional impact of this upregulation was demonstrated by the enhanced binding of EC to hyaluronate after pretreatment with bFGF. In a next set of studies to unravel the regulation of CD44 expression on EC we concluded that CD44 is an activation antigen on human EC since (1) HUVEC, which *in vivo* do not express CD44, begin to express CD44 when plated and cultured, (2) CD44 expression is enhanced after subculture of confluent cultures, (3) CD44 is predominantly expressed on the BrdU incorporating subset of cultured EC. The expression of CD44 on tumor EC prompted us to study CD44 as an endothelial target for therapy with immunotoxins. *In vitro* experiments demonstrated that EC are efficiently killed after targeting immunotoxin to CD44.

#1946 Function of MIA in metastasis of malignant melanoma. Bosserhoff, A.K., Hein, R., Wach, F., and Buettner, R. *Departments of Pathology and Dermatology, University of Regensburg Medical School, D-93042 Regensburg, Germany*

MIA (melanoma-inhibitory-activity) has been identified as a small protein secreted from malignant melanoma cells. Here we show that MIA interferes specifically with attachment of melanoma cells to fibronectin, laminin and tenascin but not to collagen type I, II and IV, vitronectin and heparansulfate-proteoglycane (HSPG). By this mechanism MIA also inhibits invasion of melanoma cells *in vitro* tested in a Boyden Chamber assay. The inhibition of attachment is due to direct molecular interaction between MIA and the matrix proteins, targeting the binding sites for integrins in this matrix proteins (RGD, XDY). Antibodies inhibiting the binding of $\alpha4/\beta1$ and $\alpha5/\beta1$ integrins to fibronectin crossreact specifically with and inactivate MIA suggesting that MIA shares significant homology with the binding pockets of these integrins and thereby masks the respective epitopes on extracellular matrix molecules. Further, enhanced MIA secretion was detected in melanoma cells grown from metastases as compared to cells grown from non-metastasized primary melanomas. In summary, we conclude that MIA plays an important role in tumor progression and spread of malignant melanomas via mediating active detachment of cells from extracellular matrix molecules within their local milieu.

#1947 The integrin $\alpha6\beta4$ and carcinoma invasion. Shaw, L.M., and Mercurio, A.M.

Mounting evidence suggests that the integrin $\alpha6\beta4$, a receptor for the laminin family of matrix proteins, is associated with carcinoma progression. However, the mechanistic basis of this association is poorly understood. Data we obtained by restoring $\alpha6\beta4$ expression in $\beta4$-deficient colorectal carcinoma cell lines indicate that a major function of $\alpha6\beta4$ is to facilitate invasion through extracellular matrices including matrix proteins that are not ligands for this integrin [Chao et al., Cancer Res. 56:4811–4819 (1996)]. The expression of $\alpha6\beta4$ is also associated with the appearance of numerous pseudopodia that may contain $\alpha6\beta4$ and specific proteases. Based on these observations, we have hypothesized that the signaling properties of $\alpha6\beta4$ enhance functions of carcinoma cells linked to invasion. To examine this hypothesis, we examined integrin signaling in MDA-MB-435 breast carcinoma cells that were engineered to express $\alpha6\beta4$. We observed that the level of protein tyrosine phosphorylation in these cells in response to integrin ligation was 5–10 fold higher than in the parent cell line that expressed $\alpha6\beta1$ and no $\alpha6\beta4$. Additional insight was provided by the finding that Matrigel invasion of these cells could be blocked by wortmannin at concentrations that are specific for inhibition of phosphoinositide 3-kinase activity. Wortmannin had no effect on the adhesion of MDA-435 cells to laminin. Taken together, our data indicate that the mechanism by which the $\alpha6\beta4$ integrin promotes carcinoma invasion involves enhanced tyrosine phosphorylation, as well as an induction of phosphoinositide 3-kinase activity. The link between these signaling mechanisms and specific cell functions associated with invasion will be discussed. [Supported by NIH CA44704 and US Army Breast Cancer Program.]

#1948 Migration arrest in glioma cells is dependent on the αv integrin subunit. Treasurywala, S., and Berens, M.E. *Neuro-Oncology Research, Barrow Neurological Institute, Phoenix, AZ 85013*

Local invasion is a hallmark of gliomas. Infiltrating tumor cells establish sites of persistent and recurrent lesions that ultimately prove fatal. Determinants of glioma cell migration include integrins and their ligands within the matrix. In contrast to the response to other matrix proteins, glioma cells migrating on tenascin do not follow a characteristic monophasic ligand density-dependent pattern. For 2 out of 4 glioma cell lines, tenascin is a permissive as well as a non-permissive substrate: i.e. low concentrations of tenascin are permissive substrates for migration, while high concentrations are non- permissive. With increasing concentrations of tenascin, glioma cells demonstrate a biphasic migration pattern. Antibody blocking studies and experiments with specific antisense oligonucleotides indicate that migration arrest is attributable to an αv containing integrin. Furthermore, cell lines that are migration arrested at high concentrations of tenascin are found to express the αv subunit, while the cell lines in our panel that migrate in a monophasic, density-dependent manner do not endogenously express αv. Loss of this integrin subunit may be associated with the invasive behavior of gliomas, suggesting that αv plays a role analogous to that of a of a tumor suppressor in the progression of gliomas to an invasive phenotype. (Supported by NIH NS27030 and Sigma Xi.)

#1949 Stromal factors influence integrin mediated oral squamous cell carcinoma adhesion and migration on tenascin-C. Ramos, D.M., Chen, B.L., Zardi, L., Sheppard, D., and Pytela, R. *University of California, San Francisco, CA 94143, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy 16132*

Tumor invasion involves multiple interactions between cancer cells and the extracellular matrix (ECM). Many ECM molecules are implicated in promoting invasion, including tenascin-C (TN-C). TN-C is transiently expressed during development and in some epithelial tumors, including oral squamous cell carcinoma (SCC). We previously demonstrated that SCC adhesion to TN-C is mediated by the αv integrin family, and now show that this adhesion involves both $\alpha2$ and αv integrins. Migration of SCC on TN-C matrices requires the presence of peritumor fibroblast (PTF) conditioned medium, and can be inhibited with antibodies to $\alpha2$ and αv integrins. A variety of growth factors have been shown to be important in promoting tumor cell motility and we show that antibodies to epidermal growth factor (EGF), hepatocyte growth factor (HGF), and transforming growth factor $\beta1$ (TGF$\beta1$) partially inhibit oral SCC migration on TN-C. We also demonstrate that invasion of complex matrices by oral SCC cells is significantly enhanced in the presence of PTF. TN-C expression can be induced in SCC cells when incubated with PTF conditioned medium, or EGF plus HGF as determined by Western blot. To evaluate TN-C matrix synthesis, SCC or PTF were grown on glass coverslips and stained with antibodies to TN-C. When grown individually, neither the SCC or the PTF organized a TN-C containing matrix. Using immunofluorescence microscopy, we identified a TN-C matrix in co-cultures of SCC and PTF. These results demonstrate the importance of mesenchymal cells in modulating oral SCC tumor cell behavior and possibly promoting invasion.

#1950 Changes in integrin expression and function associating with the ability of human non-small cell lung cancer cells to metastasize. Takenaka, K., Shinoda, K., Shibuya, M., Hibino, S., Takeda, Y., Gemma, A., and Kudoh, S. *Nippon Medical School, Tokyo, Japan*

Integrins are the major receptors by which cells attach to extracellular matrix and play an important role in cancer metastasis. To investigate the relation of integrins and metastasis, we established a highly metastatic human non-small cell lung cancer (NSCLC) cell line in nude mice, designated PC9/F9, by repeated intravenous injection of parent cell (PC9). In cell adhesion assay, PC9 adhered to laminin but PC9/F9 adhered more strongly. Furthermore, PC9/F9 adhered to type IV collagen and fibronectin. FACS scan analysis showed expression of VLA-2, VLA-3 and VLA-6 in PC9, VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 in PC9/F9. In adhesion inhibition assay, adhesion of PC9 to laminin was inhibited by anti-human $\alpha3$ and $\beta1$ integrin monoclonal antibody (Mo-Ab), on the other hand, adhesion of PC9/F9 to laminin was not inhibited by anti-$\beta1$ integrin Mo-Ab and any of anti-α integrin Mo-Ab. Adhesion of PC9/F9 to type IV collagen was inhibited by anti-$\alpha2$ and anti-$\beta1$ integrin Mo-Ab. Adhesion of PC9/F9 to fibronectin was inhibited by anti-$\alpha5$ and anti-$\beta1$ integrin Mo-Ab. Anti-$\alpha4$ Mo-Ab moderately inhibited adhesion of PC9/F9 to fibronectin. These data suggest that $\beta1$ integrins enhance the ability of human NSCLC cells to metastasize by changing their expression and function.

#1951 Is $\alpha5$ integrin a tumor suppressor gene? Taverna, D., Bronson, R.T., Ullman-Cullerè, M., and Hynes, R.O. *Massachusetts Institute of Technology, Center for Cancer Research, E17.2.24, 77 Massachusetts Avenue, Cambridge, MA 02139, USA*

One striking aspect of the phenotype of transformed cells is their reduced adhesion to solid substrates. In culture many transformed cells fail to spread as extensively as do normal cells and grow as multilayered foci. This phenomenon is probably correlated to the capability of tumor cells to invade and metastasize in vivo. In vitro data show that transformed cells fail to assemble a fibronectin-rich matrix and in some cells addition of exogenous fibronectin (FN) can revert the transformed phenotype. Loss of FN also correlates quite well with tumorigenicity and malignancy in vivo. In some cases transformed cells continue to synthesize and secrete functional FN but fail to assemble it into an ECM suggesting an alteration of cell-surface receptors for matrix molecules. Indeed, in vitro data show that there is a reduction in the level of $\alpha5\beta1$ integrin (a FN receptor), and two

other integrins in ras transformed cells. Moreover when transformed CHO (Chinese Hamster Ovary) cells overexpress human α5 and β1 integrins they were becoming non tumorigenic in nude mice. To study the importance of the FN/α5 integrin interaction in in vivo tumor formation and/or progression we used α5 and FN genetically manipulated mice (knock out). Since the null mice are not viable, in both cases, heterozygous animals (α5 -/+ and FN -/+), which showed a decreased level of α5 or FN, were compared with wild-type animals for survival and tumor formation. 50% of animals of each group died so far (over 2 years) without showing any difference in survival. In all three groups few animals developed tumors (sarcomas and carcinomas) and two α5 heterozygous animals developed an early squamous cell carcinoma of the ear canal. Moreover we analyzed whether a decreased level of α5 or FN expression could affect tumorigenicity in mice that develop a high rate of tumors by crossing p53 null animals (kindly provided by T. Jacks, MIT) with α5 and FN heterozygous mice. p53 is a tumor suppressor gene that appears to be a critical regulator of normal cell proliferation and also causes apoptosis in cells with genomic damage. Mutant alleles of this gene are in fact found in many human tumors. The p53 knock out animals are viable and fertile but they are highly prone to malignancy in the first six months after birth. The heterozygous animals (p53 -/+) also develop tumors but later in their life (9–17 months). The following 4 experimental groups were monitored and compared to control animals: p53 null, α5 heterozygous; p53 null, FN heterozygous; p53 heterozygous, α5 heterozygous; p53 heterozygous, FN heterozygous. Most of the α5 (or FN) -/+, p53 -/- died at 3 up to 9 months because of tumor formation. Lymphomas, fibrosarcomas, hemangiosarcomas or osteosarcomas were observed. 20% of the α5 -/+, p53 -/- animals developed cataracts in one or both eyes. No cataract was observed in control animals.The double heterozygous animals died between 6 to 23 months. They developed fibrosarcomas, hemangiosarcoma, osteosarcomas, rhabdomyosarcoma, lymphomas, squamous cell carcinomas and adenocarcinomas. Few animals developed metastases. The survival and the pattern of tumour formation or progression of the four groups was very similar to our p53 control mice (null or heterozygous). A third approach was then undertaken: we generated "chimeric mice", i.e. animals containing α5 null cells mixed with α5 positive cells. We are in the process of monitoring these animals and the results will be discussed. The results obtained with the knock out animal study indicate that a decrease in α5 or FN expression in mouse does not play a role in tumorigenesis and metastasis formation. These results are in conflict with the implications of previous in vitro observations. The data from the chimeric animals will be indispensable to confirm or deny the role of α5 integrin as tumor suppressor gene.

#1952 Combined IFN-γ and TNF-α treatment differentially affects adhesion and migration of keratinocyte-derived cells to laminin. van den Brûle, F.A., Delvenne, P., Clausse, N., Franzen, E., and Castronovo, V. *Metastasis Research Laboratory and Dept. of Pathology, University of Liegè, Belgium*

Interactions between cells and basement membrane glycoprotein laminin are key events during cancer progression. In this study, we examined the abilities of three human keratinocyte-derived cell lines (two HPV DNA transformed cell lines, 18–11S3 and EIL8, and the carcinoma-derived, HPV DNA-containing SiHa cell line) to attach and migrate to laminin before and after a combined IFN-γ and TNF-α treatment. SiHa cells adhere much better to laminin than EIL8 and 18–11S3 cells (± 50 vs. 20–30% attached cells at 60 minutes, respectively). Preliminary data show that combined treatment of the cells with the cytokines during 24 hours, at doses that significantly decrease cell proliferation, did not significantly affect adhesion in any of the cell lines. Interestingly, chemotaxis experiments demonstrated that only SiHa cells did significantly migrate to soluble laminin compared to 18–11S3 and EIL8. The combined cytokine treatment did not affect the 18–11S3 and EIL8 cell motile response but significantly decreased the migration of SiHa cells to laminin. In conclusion, we demonstrate a selective effect of IFN-γ and TNF-α treatment on the chemotactic, but not on the adhesive abilities of HPV-containing SiHa cells, to laminin. Our data could help understanding the biologic effects of combined IFN-γ and TNF-α treatment.

#1953 Expression of the 67-kD elastin binding protein in human gliomas: Implications for glioma invasion. Jung, S., Hinek, A., and Rutka, J. *Divisions of Neurosurgery and Cardiovascular Research, The Hospital for Sick Children, Toronto*

Elastin is present in significant amounts in the cerebral microvasculature of the normal human brain. However, the role that elastin plays in either facilitating glioma attachment, or inhibiting glioma invasion, has not been well characterized. Therefore, we analyzed 7 human astrocytoma cell lines for their production of the 67-kD elastin binding protein (EBP) by immunocytochemistry and western blot, for their attachment to elastin *in vitro*, for their migration through an intact elastin membrane, and for their ability to degrade tritiated-elastin. All cell lines expressed the 67-kD EBP and attached to elastin *in vitro*. There were three cell lines, U 87 MG, U 373, and SF-188, which expressed high levels of the 67-kD EBP. All cell lines attached to elastin *in vitro* with U 87 MG showing the greatest degree of adhesion. All cell lines except SF-539 were capable of penetrating and migrating through an intact elastin membrane. All cell lines were capable of degrading tritiated-elastin, a process which could be prevented by the incubation of glioma cells with EDTA. Our data show for the first time that glioma cells express a functional 67-kD EBP enabling them to recognize and attach to elastin as a substrate. In addition, our results show that glioma cells possess significant elastolytic activity. Ongoing studies are determining the role of the 67-kD EBP and elastolytic enzymes in gliomas *in vivo*.

#1954 Supression of adhesion between laminin and endometrial adenocarcinoma cells by β1–4 galactosyltransferase gene introduction. Kubushiro, K., Tsukazaki, K., Ma, J., Mikami, M., and Nozawa, S. *Keio University, Tokyo, Japan 160*

Glycoconjugates on cancer cells play an important role in the process of metastasis. We have found that endometrial cancer expressed mainly type I carbohydrate chains. Furthermore, endometrial cancer with a poor prognosis tended to show little expression of Lewis^b, one of type I carbohydrate chain. It is well known β1–3 galactosyltransferase (β1–3GT) and β1–4 galactosyltransferase (β1–4GT) are involved in the synthesis of type I and type II carbohydrate chain, respectively. In order to clarify the biological function of the type I chain in endometrial cancer, we transfected human β1–4GT cDNA into endometrial cancer cell line and studied the biological function between transfectants and SNG-M. Endometrial cancer cell line, SNG-M cells were confirmed to express low levels of β1–4GT protein and mRNA by western blotting and northern blotting. Then β1–4GT cDNA inserted expression vector pCAGGS was transfected into SNG-M cells. Using these transfectants, the adhesiveness to laminin was examined by vitro adhesion assay. Five clones showing greater expression of β1–4GT mRNA and proteins were obtained. These five transfectants showed greater adhesiveness to laminin compaired to original SNG-M cells. It is suggested that endometrial cancer cell lines with increased expression of β1–4GT could have different biological function compared with original cell line.

#1955 Surface labelling of tumor cells with a photoaffinity probe based on the peptide 11 sequence. Starkey, J.R., Berglund, D., and Kazmin, D.A. *Montana State University, Bozeman, MT 59717*

Laminin peptide 11, CDPGYIGSR, can block tumor cell invasion and metastasis. It is the putative primary ligand for the 32/67 kDa laminin binding protein (LBP), however, interactions between the peptide 11 and cell surface molecules such as the 32/67 kDa LBP are poorly understood. To approach this problem, we synthesized a highly specific probe by replacing the tyrosine in peptide 11 with the photocrosslinker, 4-benzoyl-L-phenylalanine. For detection, we added a biotinylated lysine at the N- terminus, and included three glycines as a spacer before the peptide 11 sequence. Tumor cells were treated with 0.05 mM probe, exposed to UV light, and incubated with FITC-conjugated avidin for FACScan analysis. Using standardized avidin-FITC, we found an average of 10^5 surface labeled sites per cell, a similar number to that previously determined using a sequence specific antibody to the 32/67 kDa LBP. We were able to visualize a single crosslinked protein on Western blots of detergent extracts from the membranes of treated tumor cells. The molecular weight of the labeled protein was consistent with the 67 kDa membrane form of the LBP modified by two copies of the probe. Finally, using confocal microscopy, we evaluated cell surface labelling patterns on different matrix substrates. This versatile new probe should greatly facilitate the elucidation of peptide 11 interactions with malignant tumor cells.

#1956 Regulation of adhesion of highly human metastatic tongue squamous carcinoma cells (SCC) to extracellular matrix by phorbol 12-myristate 13-acetate and calphostin C. Xue, H., White, F.H., Tipoe, G.L., Wu, J.Z., and Situ, Z.Q. *Department of Anatomy, The University of Hong Kong, Hong Kong, Department of Oral Biology, The Fourth Military Medical University, Xian, P.R. China*

Adhesion molecules expressed on the tumor cell surface not only mediate cell-cell and cell-extracellular matrix (ECM) attachment, but also are signal transducers, which may transmit certain signals to tumor cells, causing abnormal function. The expression and function of these adhesions are modulated by many factors including intracellular signal transduction. Protein kinase C (PKC) is a family of closely related lipid-dependent isoenzymes involved in intracellular signalling during cell differentiation and proliferation. This study investigates the roles of phorbol 12-myristate 13-acetate (PMA), an activator of PKC and calphostin C, an inhibitor of PKC on the adhesion to ECM of a human tongue SCC cell line highly metastatic to the brain (TmB). PMA significantly enhanced TmB cell adhesion to four kinds of ECM (vitronectin, collagen IV, fibronectin and laminin) when compared with the control group. This enhancement of TmB cell adhesion to the four ECM components was dose-dependent from 10^{-11} to 10^{-7} mol/L. However, 10^{-6} and 10^{-5} mol/L slightly reduced TmB cell adhesion to the four kinds of ECM when compared with 10^{-7} mol/L. No significant differences were detected in cell adhesion of TmB between the four ECM components after treatment with PMA. PMA induced optimum effects after 45min incubation. However, pretreatment of calphostin C (1.0 μmol/L) dramatically blocked the stimulation of adhesion to ECM by PMA. PMA and calphostin C may regulate cell-ECM adhesion, which itself may be mediated by PKC activation.

#1957 Neural cell adhesion molecule (NCAM) plays a role in the invasiveness of CNS-1 rat glioma. Owens, G.C., DeMasters, B.K.K., Savelieva, E., Withrow, C., Orr, E.A., and Kruse, C.A. *Univ. of Colo. Health Sci. Ctr., Denver, CO 80262*

Thrombosis and Haemostasis © F.K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 78(1) 83–87 (1997)

Targeted Mutations in Integrins and their Ligands: Their Implications for Vascular Biology

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Adhesion of cells to one another and to the underlying or surrounding extracellular matrix plays crucial roles in the development, physiology and pathology of all metazoans. This is as true in the vasculature as anywhere else. Indeed, cell adhesion has been most effectively studied in the context of vascular biology; we know more about cell adhesion of vascular cells than about any others and the concepts developed there serve as models for thinking about cell adhesion in other contexts (1).

During development of the vasculature, cell adhesion plays roles in the assembly of the heart and vessels; the cells need to migrate to the correct locations and assemble into tubes, which need to be arrayed correctly. These morphogenetic events rely on cell adhesion; both cell-cell and cell-matrix. If adhesion is improperly regulated during development, abnormalities in the heart or blood vessels can ensue.

During normal functioning of the vasculature, circulating cells such as lymphocytes need to adhere in appropriate locations during their traffic around the body. In response to infection or injury, leukocytes need to adhere and extravasate at sites of infection and platelets need to adhere to damaged vessel walls and to each other to prevent bleeding. When these adhesion processes are defective, disease results; susceptibility to infections or bleeding, respectively. While defective adhesion of blood cells is deleterious, so is excessive adhesion. Inappropriate adhesion of leukocytes or platelets can lead to inflammation or thrombosis, respectively. Therefore, adhesion must be tightly regulated to give sufficient adhesion when needed and to prevent inappropriate or excessive adhesion.

We now understand quite a bit about the adhesion receptors mediating these events and some inferences can be made about how adhesion is regulated (1,2). There are dozens of cell surface receptors contributing to cell adhesion in one system or other. Fortunately many of them fall into a few families. The major families of receptors involved in cell-cell adhesion are members of the *immunoglobulin superfamily* (IgSF) and *cadherins*, both of which can mediate homophilic (like with like) adhesion between cells. IgSF members can also function in heterophilic interactions (between unlike molecules) with members of another family of adhesion receptors, the *integrins* (1–3). IgSF/integrin interactions are important in many instances of blood cell-vessel wall adhesion (see below). Another important set of heterophilic interactions is that between *selectins*, carbohydrate-binding lectins, and their counterreceptors, glycoproteins presenting the relevant carbohydrate recognition motifs (4,5). The main family of cell-matrix adhesion receptors are the *integrins*, and, in this review, we will concentrate on them and, in particular, on what has been learned from studies of mice with mutations in the genes encoding integrins and their ligands (2,6,7).

Integrins in vascular biology

There are around twenty known integrins, each of which is a heterodimer of α and β subunits. There are currently 16 α subunits and 8 β subunits known, but not all combinations occur and not all the 20 which do are relevant for vascular biology (Fig. 1). There is degeneracy in the interactions of

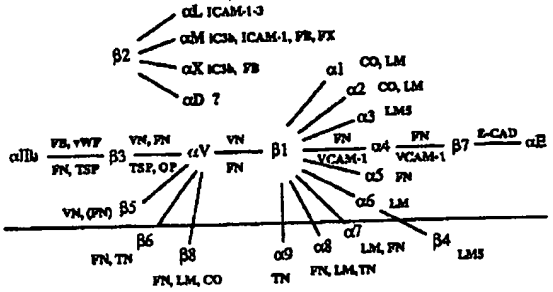

Fig. 1. Integrins of potential relevance in vascular biology. The figure depicts the αβ pairings and major ligand specificities of integrins (2). Those integrins below the line are not known to play any part in the vascular system but are included here for completeness.
Abbreviations: CO, collagen(s); E-cad, E-cadherin; FB, fibrinogen; FN, fibronectin; LM, laminin; OP, osteopontin; TN, tenascin-C; TSP, thrombospondin-1; VN, vitronectin; vWF, von Willebrand factor; iC3b, inactivated C3 component of complement; FX, factor X.

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integrins with their ligands; multiple integrins can recognize a given ligand and many integrins can recognize multiple ligands.

Among the integrins, a subset participates in heterotypic cell-cell adhesion and all these play important roles in vascular biology (2,3). This subset includes the β2 integrins, which bind IgSF molecules such as ICAMs1-3, the two α4 integrins (α4β1 and α4β7) which recognize the IgSF counterreceptor VCAM-1 (α4β7 also recognizes another cell surface receptor, MAdCAM-1), αvβ3 which is reported to bind to CD31/PECAM-1 (8), and αEβ7, which is only expressed by intraepithelial lymphocytes and binds E-cadherin on epithelial cells (9,10). These cell-cell adhesion-mediating integrins are widely expressed on circulating blood cells and their counterreceptors are expressed on many cell types, including other leukocytes and endothelial cells. The platelet-specific integrin αIIbβ3 also mediates cell-cell adhesion of platelets, by binding the dimeric molecule fibrinogen which can bridge between adjacent platelets (11). The other integrins are concerned with cell-matrix adhesion and many of them , especially β1 and β3 integrins, are expressed on platelets, leukocytes and endothelial cells.

It is clearly a challenge to assign functions to individual integrins in a given vascular adhesion process; cells frequently express multiple integrins with partially overlapping ligand specificities. Various approaches have been taken to address such questions. Much useful information has been gained using antibodies specific for given integrins, their counterreceptors or ligands to block specific adhesion events. Indeed this is how many integrins were first identified. Similarly, those integrins which recognize the sequence RGD in their ligands (the αv and β3 integrins and α5β1) can all be blocked by peptidomimetics based on this sequence. Furthermore, peptidomimetics selective for given integrins in this group have been developed and have been useful in probing their involvement in specific processes (12). Similar approaches have been used to a lesser extent for α4 integrins but have not yet been particularly useful for other integrins, whose binding sites have not been so precisely defined.

Another obvious approach would be to use genetic approaches to ablate specific integrin subunits. In fact, two human genetic diseases have integrin genes as their targets and provided crucial early evidence for the importance of integrins in vascular biology. The bleeding disorder, Glanzmann's thrombasthenia (GT) arises from genetic defects in the genes for the integrin, αIIbβ3, the major fibrinogen receptor on platelets, which plays a central role in hemostasis (11). Studies of αIIbβ3 integrin and of the mutations leading to GT have played an important role in our understanding of integrin function. Similarly, the rare genetic disease, leukocyte adhesion deficiency I, affects the gene for the β2 integrin subunit. LAD I patients lack β2 and, therefore, all β2 integrins and their leukocytes are severely compromised in adhesion and extravasation at sites of infection (13). So far, there are few reports of human mutations affecting other integrin subunits, perhaps because they result in embryonic lethality (see below). An exception is the report of a defect in α2β1 integrin affecting platelet function and leading to a bleeding disorder (14) but the actual genetic defect has not been defined. Well known examples of genetic diseases affecting integrin ligands of importance to vascular function are afibrinogenemia and von Willebrand's disease (15).

Useful as these human mutations have been, they have significant limitations. They are rare diseases and there are obvious limits on the experimental analyses that can be performed on the affected patients. Many integrins lack known human genetic defects and it is not possible to combine even those that are known with other mutations. It would be invaluable to our understanding of integrin functions to have an experimental system allowing genetic analyses of any integrin or combination of integrins. Fortunately, technical advances in the past decade have made possible such genetic manipulations in mice and these have provided much valuable information about the functions of integrins and their ligands in the development and functioning of the vasculature.

Murine mutations in integrins and their ligands

In recent years, "knockout" or null mutations have been made in the genes for most integrins (see Table 1; refs 6,7). Among

Table 1. Integrin Knockout Mutations

β1	Early embryonic lethality. No vasculature
α1	Viable. No obvious vascular defects
*α2	ND
α3	Perinatal lethality. Defects in kidneys, lung and skin. No obvious defects
*α4	Defects in placentation, heart development and lymphocyte and homong
*α5	Embryonic lethality. Defects in embryonic and extraembryonic vasculature
α6	Perinatal lethality. Major skin defects. No obvious vascular defects
α7	Viable. Muscular dystrophy. No reported vascular defects
α8	Perinatal lethality. Defects in kidney development. No reported vascular defects
α9	Early postnatal death; chylothorax
*αv	Embryonic and perinatal lethality. Hemorrhage
*αIIb	ND
*β3	Viable
β4	Perinatal lethality. Major skin defects. No obvious vascular defects
*β5	Viable. No obvious defects
β6	Viable. Inflammation. No obvious vascular defects
β8	ND
*β7	Viable. Defects in gut-associated lymphoid tissue
*αE	Viable. Some defectsin gut-associated lymphoid tissue
*β2	Viable. Defects in inflammatory responses and trans plant rejection
*αL	Viable. Defects in mixed lymphocyte response and allograft rejection
*αM	Viable. Defects in neutrophil apoptosis
*αX	ND
*αD	ND

*marks integrins of relevance to vascular biology.
ND = not done
References are given in the text for those integrins of known relevance to vascular biology. For other references see 6,7.

integrins of potential relevance for vascular biology (Fig. 1) only α2, αIIβ, αX and αD remain unscathed. Equally, many of the relevant extracellular matrix ligands and counterreceptors have been "knocked out" (6).

What have we learned from these mutations? One set of mutations has major effects on vascular development and are lethal mutations. These include mutations in fibronectin and two of its receptor integrins, α5β1 and αv. Fibronectin-null embryos die 2-3 days after implantation with heart defects and defective vasculature, both within the embryo itself and in the yolk sac (16,17). The severity of the defects depends on the genetic background and lies in the execution of cardiogenesis and vasculogenesis (18). The progenitors of cardiac myocytes and endothelial cells are induced at the correct times and places but, to one degree or another, fail to form heart and vessels. The α5-null mutation produces similar but milder defects (19), clearly indicating that another fibronectin receptor must be operating in addition to α5β1. Analyses of α5-null cells suggested that αvβ1 could substitute for α5β1 (20). Furthermore, αv integrins (αvβ3 and αvβ5) have been implicated in vasculogenesis and angiogenesis (21). For these reasons, we ablated the αv gene (Bader and Hynes, unpublished data). Surprisingly, there was extensive development of the heart and vasculature. Around 20% of αv-null progeny develop to term, are born alive but die soon afterward. These pups show hemorrhage in the brain and intestine and further analyses of the αv-null embryos and pups should provide valuable insights into the roles of αv integrins in vascular development. One interpretation would be that much of cardiogenesis and vasculogenesis is αv-independent but that development and/or maintenance of the brain vasculature does depend on αv integrins. It is possible that different forms of angiogenesis exist, which differ in their requirements for av integrins. It is interesting to note that knockouts of β3, β5 and β6 are all viable with no obvious defects in vascular development (Table 1), although more analyses are needed to investigate these issues further and double mutants will need to be generated. It is, furthermore, of some interest that knockouts of most ligands for αv integrins yield viable mice (6); something of a surprise. Overall, the effects of mutations in av integrins or their ligands are all milder than might have been anticipated. This shows that many of these proteins are not *essential* for development, although overlapping or subtle functions are not yet ruled out. It seems plausible to suggest that this set of receptors and ligands may have evolved to play roles in repair processes.

As discussed, αv integrins may be angiogenic in some situations. In contrast, it appears that thrombospondin-1 (TSP) can act as a suppressor of angiogenesis (22,23). TSP can be a ligand for αvβ3 and αIIbβ3 integrins, although it also has other receptors (23). Cells from TSP-null mice show a reduction in their ability to suppress angiogenesis (24). Thus, further analyses of αv and TSP mutant mice should provide insights into the regulation of angiogenesis.

In contrast with the milder than expected phenotype of av integrin mutations, mutations in α4 integrin and VCAM-1 are more deleterious than anticipated (25–27). It had been thought that α4 was predominantly expressed on circulating blood cells and that VCAM-1 (vascular cell adhesion molecule 1) was solely expressed by endothelial cells. α4/VCAM-1-mediated adhesion was thought to be involved primarily in adhesion of lymphocytes, monocytes and eosinophils to inflamed endothelium. However, when α4 and VCAM-1 were knocked out, the phenotypes of the resulting mutants revealed unexpected roles for α4/VCAM-1 interactions in vascular development. Specifically this interaction plays a role in chorio-allantoic adhesion to form the placenta and in the development and maintenance of the epicardium and coronary vessels (25–27). Because of these defects, α4-null embryos die in utero (25) as do the vast majority of VCAM-1-null embryos (26,27).

Apart from FN, α4, α5, αv and VCAM-1, other integrins and their ligands do not appear to play major essential roles in vascular development (see Table 1 and refs 6 and 7). Mutations in several other integrins and ligands do have effects on vascular functions in adult animals. Those integrins which appear to be expressed only by white blood cells (α4β7, αEβ7, β2 integrins) might be expected to be dispensible for development and that appears to be the case (see lower part of Table 1). This set of mice does however shed light on the roles of integrins in leukocyte traffic. Absence of α4β7 and αEβ7 or of αEβ7 alone leads to deficits in recruitment of lymphocytes to the gut-associated lymphoid tissue (28,29), consistent with a role of these integrins in homing to those sites. Similarly, analyses of animals chimeric for the expression of α4 integrins reveals a role for these integrins in homing to Peyer's patches but not to the intestinal epithelium (30) and a similar analysis of β1 integrin chimeras reveals a role for some β1 integrins (but not α4β1, ref 30) in seeding the fetal liver with lymphoid precursors (31). The α4 chimeras also show that both B and T cell progenitors need α4 integrins for normal development in the bone marrow, though apparently not in the fetal liver or thymus (30). Further work along these lines should uncover other roles for specific integrins in the development and/or homing of distinct lymphoid and myeloid cells.

The mutations in β2 integrin genes and in ICAM-1 also provide information about myeloid cell traffic. As expected from the human LAD I patients, β2-deficient mice show reduced recruitment of neutrophils to sites of inflammation (32). The same is true of ICAM-1-deficient mice (33,34). However, since these mutations appear to be hypomorphic rather than null (32,35), the defects are not complete. It will be of interest to determine how the severity of the defects is altered as true null mutations are analyzed. Furthermore, the possibility of overlapping roles of the several ICAMs will need to be addressed by the ablation of the other genes and the generation of double mutations.

Ablation of the αL gene produced defects in mixed lymphocyte response (as did the ICAM-1 mutation) in conformity with expectation from in vitro analyses and also showed deficits in allograft rejection and in responses against tumors (36,37). In contrast, mice lacking αM showed a marked increase in neutrophils in a chemical peritonitis model and it was found that αMβ2 is important for phagocytosis-induced apoptosis; an unexpected result (38). Further analyses of these mice should be informative concerning the

roles of the different β2 integrins and will allow deeper analyses of the defects in LAD I patients.

Similarly, recently obtained strains with null mutations in β3 integrin (39) and von Willebrand factor (40) will serve as mouse models for the human bleeding disorders, Glanzmann's thrombasthenia and von Willebrand's disease. The availability of such mouse models will allow experimental analyses not feasible with human patients and should provide new insights into those diseases and, likely, new therapies.

Conclusions

The development of strains of mice defective in specific integrin subunits or in their ligands or counterreceptors has already provided new information about their roles in the development of the vasculature and in the behavior of blood cells. Further analyses of these mice and of cells there from, as well as the generation of mice with more subtle defects in the same genes, promise to provide further insights and valuable animal models of human disease states including inflammatory disorders and defects in thrombosis and hemostasis.

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Increased primary tumor growth in mice null for β3- or β3/β5-integrins or selectins

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Expression of αvβ3- or αvβ5-integrins and selectins is widespread on blood cells and endothelial cells. Here we report that human tumor cells injected s.c. into mice lacking β3- or β3/β5-integrins or various selectins show enhanced tumor growth compared with growth in control mice. There was increased angiogenesis in mice lacking β3-integrins, but no difference in structure of the vessels was observed by histology or by staining for NG2 and smooth muscle actin in pericytes. Bone marrow transplants suggest that the absence of β3-integrins on bone marrow-derived host cells contributes to the enhanced tumor growth in β3-null mice, although few, if any, bone marrow-derived endothelial cells were found in the tumor vasculature. Tumor growth also was affected by bone marrow-derived cells in mice lacking any one or all three selectins, implicating both leukocyte and endothelial selectins in tumor suppression. Reduced infiltration of macrophages was observed in tumors grown in mice lacking either β3-integrins or selectins. These results implicate cells of the innate immune system, macrophages or perhaps natural killer cells, in each case dependent on integrins and selectins, in tumor suppression.

tumors | bone marrow transplants | cell adhesion | innate immunity

We have previously investigated the growth and metastasis of transplanted tumors in mice lacking specific cell-adhesion receptors, namely integrins (1–3) or selectins (4–6).

In mice lacking αv-integrins (αvβ3 and αvβ5), tumors grow larger; this growth is accompanied by enhanced tumor angiogenesis (2). However, it is unclear whether other factors, such as altered vessel morphology, pericyte recruitment, or altered immune responses in the integrin-deficient mice, may contribute to the enhanced tumor growth.

In the case of selectins, we have shown that experimental metastases to the lungs are reduced in mice lacking P- and/or L-selectins (4–6). This reduction is believed to result from the tumor cells' expression of ligands for selectins such that selectins on host platelets, leukocytes, or endothelial cells can bind these ligands and promote metastatic arrest in the lungs after tail vein injection (7–10). Examination of this hypothesis using a more complete model of metastasis, such as metastasis from a s.c. site, would be desirable.

Given these two prior lines of investigation and the questions they raised, we have investigated further the s.c. growth of xenotransplanted tumors in mice lacking various combinations of integrins or selectins. We report here results indicating that both β3-integrins and selectins contribute to host responses suppressing tumor growth. In each case, bone marrow (BM)-derived host cells, dependent on integrins or selectins, seem to inhibit tumor growth, and in the absence of these adhesion receptors tumors grow significantly larger.

Materials and Methods

Mice. All mice were generated in our own laboratory. *β3-Integrin*-null mice (2, 11–12) were intercrossed with mice lacking the *Rag2* gene (13) and with mice lacking β5-integrin (14) to generate mice deficient in *Rag2*, in *Rag2* and *β3-integrin*, or in *Rag2*, *β3-*, and *β5-integrin* genes. Mice lacking all combinations of the three selectin genes (P-, L-, and E-selectins; ref. 15) were crossed with the *Rag2*-null mice to generate mice deficient in *Rag2* and various combinations of selectins. All mice used in the experiments described here were, therefore, *Rag2*-null on a mixed C57BL/6 × 129S4 background and lacked B, T, and NKT cells. Because of the complexities of the breeding, experimental mice having or lacking individual adhesion genes were cousins, not siblings. However, the effects seen were consistent among diverse experiments and highly significant. Mice were maintained under severe combined immunodeficient conditions in the Massachusetts Institute of Technology animal facility. All mice were viable and fertile and generally remained healthy for the duration of the experiments, despite occasional hemorrhage in β3-integrin-deficient mice (11) and late-onset dermatitis in mice lacking both endothelial selectins P and E (15, 16). In an earlier study of metastasis, tumor growth was reduced in P-selectin-deficient mice (4), but those mice were not kept in specific pathogen-free conditions and showed opportunistic infections and significant morbidity that apparently affected tumor growth.

Antibodies. Monoclonal antibodies used for immunohistochemistry were rat anti-platelet endothelial cell-adhesion molecule (PECAM)-1/CD31, rat anti-Pan-NK, biotinylated hamster anti-β3-integrin (all from PharMingen), mouse anti-smooth muscle α actin (Sigma), rat anti-F4/80 (Serotec), and rabbit anti-NG2 polyclonal antiserum (Chemicon); all were used at a 1:100 dilution. Secondary antibodies for immunofluorescence were Alexa Fluor-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-rat IgG (all from Molecular Probes). Biotin-conjugated goat anti-rat Ig antibody used for chromogenic analysis was from PharMingen. Fluorescence-activated cell sorter analyses used FITC-conjugated rat monoclonal antibodies anti-Pan-NK or anti-F4/80.

Immunohistochemistry and Histology. Five-micrometer frozen sections of tumors were used for immunohistochemistry. Chromogenic visualization followed the VECTASTAIN ABC kit protocol (Vector Laboratories). For immunofluorescence, the conjugated secondary antibodies were used at a 1:400 dilution. Histology was performed on 5-μm paraffin-embedded or 1- to 2-μm plastic-embedded tumor sections counterstained with hematoxylin/eosin or with methyl green, respectively. All images were visualized with a Zeiss Axiophot photoscope. Images were

Abbreviations: BM, bone marrow; PECAM-1, platelet endothelial cell-adhesion molecule 1.

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processed with NIH SCION IMAGE and ADOBE PHOTOSHOP (Adobe Systems, Mountain View, CA).

Flow Cytometry. FITC-conjugated rat monoclonal antibodies (anti-mouse Pan-NK or anti-F4/80) were used to stain natural killer (NK) cells or monocytes/macrophages present in the blood of mice. Blood samples were incubated with a 1:100 dilution of antibody for 1 h at room temperature, treated with red cell lysis buffer (17), washed twice with PBS, resuspended in PBS, and analyzed in a FACScan with CELLQUEST software (Becton Dickinson).

Mouse Tumor Transplants. Eight- to 12-week-old mice were injected s.c. with either 2×10^6 LS 180 human colon cancer cells (ATCC no. CL-187) or 2×10^6 A375 SM human melanoma cells (18). Tumors were harvested, weighed, and processed from 3 days to 5 weeks later. Every experiment was repeated at least 3 times.

Mouse BM Transplants. Eight-week-old mice (recipients) were lethally irradiated with 1,000 rads (a dose of 600 rads followed 4 h later by a dose of 400 rads; 1 rad = 0.01 Gy) and reconstituted with an intraorbital injection of 2×10^6 BM cells from donors of the desired genetic makeup. These mice were kept in sterile conditions and fed antibiotics for 10 days. Five weeks after the BM transplant, mice were injected s.c. with human tumor cells and analyzed as described above.

Statistical Analysis. Data were analyzed by using a program available at www.physics.csbsju.edu/stats. Tumor sizes are represented by box-and-whisker plots (Figs. 1, 3, 5, and 6) wherein each box represents an interquartile range (IQR; the central 50% of the data points), the horizontal line in each box represents the median, and vertical bars represent a spread of 1.5 × IQR. Dots represent outliers, which were included in calculations of significance; n indicates the number of samples, and p indicates the probability of identity of the distributions. In Fig. 5*a*, data from several experiments were combined and expressed as percentages of the mean WT value, and the box-and-whisker plots were generated by using EXCEL (Microsoft) (19).

Results

Tumor Growth Is Enhanced in β3- or β3/β5-Null Mice. Immunocompromised *Rag2*-null (control), *Rag2*/β3-null, and *Rag2*/β3/β5-null mice were injected s.c. with human tumor cells, either colon carcinoma (LS 180) or melanoma (A375 SM). As shown in Fig. 1, tumors grew in all three lines of mice, but tumor size was enhanced significantly in *Rag2*/β3- or *Rag2*/β3/β5-integrin-deficient mice when compared with *Rag2*-null mice WT for integrins ($P < 0.0001$) Differences in growth rates were seen as early as 2 weeks after implantation.

Angiogenesis Is Not Blocked in the Absence of β3- or β3/β5-Integrins. Sections of human tumors were analyzed at different times after the s.c. injections. No morphological differences were observed among the vessels within the different tumors. Immunohistochemical analysis with anti-PECAM-1, an endothelial cell marker, revealed an increased blood vessel density in the absence of β3-integrins (Fig. 2) or β3/β5-integrins (data not shown), as previously described for other tumors (2, 18). To analyze possible differences in the recruitment of vascular mural cells (pericytes and smooth muscle cells), sections of tumors were stained with antibodies against NG2 (Fig. 2) or smooth muscle α actin (data not shown), markers for pericytes, and costained with anti-PECAM-1 antibody to reveal the endothelial cells (Fig. 2 *Left*). Similar staining for NG2 or smooth muscle α actin was observed around the vessels of tumors grown in control or in β3- or β3/β5-null mice. Thus, the absence of these integrins does not block vessel development, which is in fact enhanced (≈17%) compared with that in the WT.

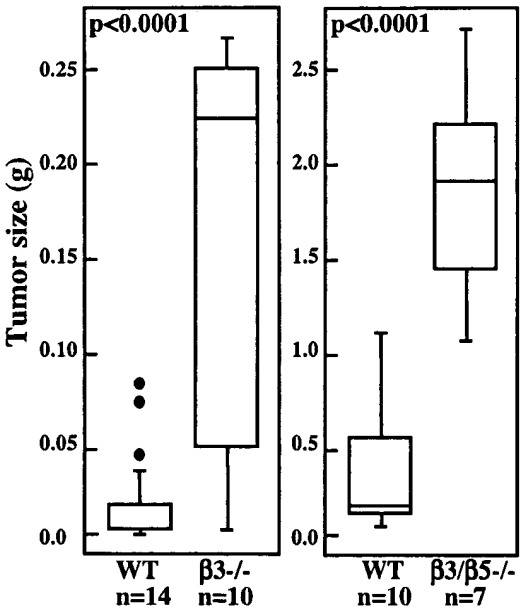

Fig. 1. Tumor growth is increased in β3- and β3/β5-integrin-deficient mice. Human A375 SM (*Left*) and LS 180 (*Right*) tumors grown s.c. in *Rag2*-null/$\beta3^{+/+}$ and *Rag2*/β3-null or *Rag2*/β3/β5-null mice. Box plots show tumor weights in grams for 4-week-old tumors (*Left*) and 3-week-old tumors (*Right*). $P < 0.0001$; n, the number of animals in each group.

Increased Tumor Growth in β3-Null Mice Is BM-Dependent. To determine whether BM cells were responsible for the different tumor growth in the various mouse lines, recipients (*Rag2*-null or *Rag2*/β3-null) were lethally irradiated and reconstituted with BM derived from mice with different genetic backgrounds. Five weeks after the BM transplants, these mice were injected s.c.

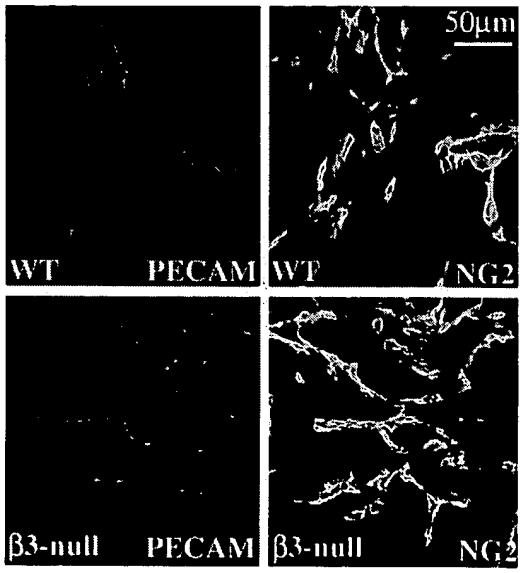

Fig. 2. Characterization of vasculature in human A375 SM and LS 180 tumors grown in *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null mice. PECAM-1 and NG2 double-staining of 11-day-old LS 180 tumors from *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null mice. PECAM-1 (red) and NG2 (green) staining are present around the same vessels. Similar results were obtained with smooth muscle α actin staining for pericytes and with tumors grown in *Rag2*/β3/β5-null mice (data not shown).

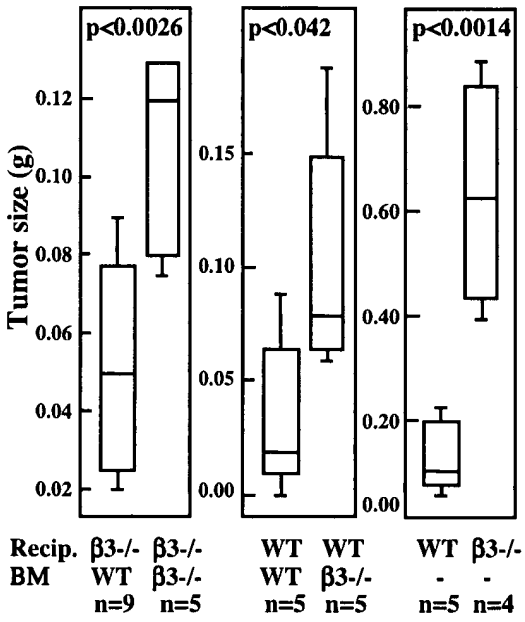

Fig. 3. Analysis of s.c. tumors in mice after BM transplantation. *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null recipient (Recip.) mice were lethally irradiated and reconstituted with BM derived from *Rag2*/$\beta3^{+/+}$ or *Rag2*/β3-null mice and were injected s.c. 5 weeks later with LS 180 human tumor cells. The box plots represent the size of 3-week-old tumors. Reduced tumor growth can be observed in mice that received *Rag2*-null/$\beta3^{+/+}$ BM compared with mice that received *Rag2*/β3-null BM. (*Right*) Nonirradiated mice that did not receive a BM transplant are shown for comparison. (*Left*) $P < 0.0026$. (*Center*) $P < 0.042$. (*Right*) $P < 0.0014$. n, Number of animals in each group.

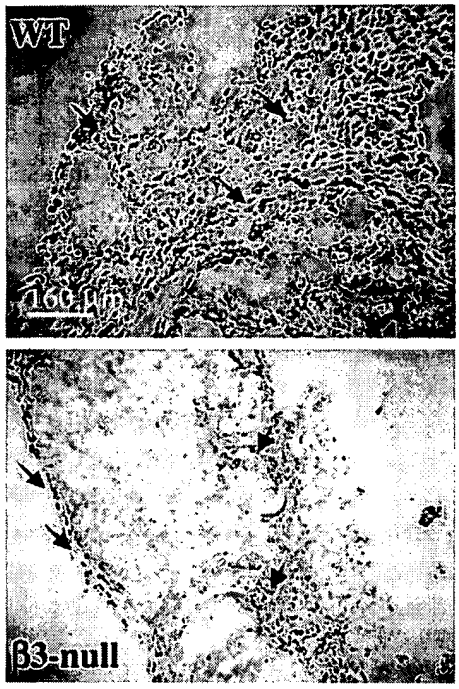

Fig. 4. Macrophage infiltration in A375 SM tumors grown in *Rag2*/$\beta3^{+/+}$ or *Rag2*/β3-null mice. Sections of 3-day-old A375 SM tumors were stained with an anti-macrophage antibody. A higher infiltration of macrophages (brown, indicated by arrows) was observed in tumors grown in control mice (*Upper*) compared with tumors grown in *Rag2*/β3-null mice (*Lower*). Note also the greater infiltration into the tumor in $\beta3^{+/+}$ mice. (Scale bar, 160 μm.)

with tumor cells. As shown in Fig. 3, tumor growth was enhanced only in mice reconstituted with BM cells derived from β3-null mice. Tumor growth was reduced in all irradiated compared with nonirradiated mice, independent of genetic makeup (note different scales). However, in all comparisons, mice with BM WT for integrins supported significantly less tumor growth than did mice with BM lacking β3-integrins. Therefore, BM-derived, integrin-dependent, host cell type(s) suppress tumor growth.

To investigate whether the increased tumor growth in animals that received BM transplants could be affected by endothelial cell precursors contributing to the excess vessel growth, we stained for β3-integrins. Tumors grown in β3-null animals showed no staining for β3-integrin on the vessels, whereas positive endothelial cell staining was observed on vessels of tumors grown in *Rag2*-null/$\beta3^{+/+}$ animals or in the same animals transplanted with $\beta3^{-/-}$ BM cells. However, when β3-null animals were transplanted with *Rag2*-null/$\beta3^{+/+}$ BM cells, no positive staining for β3-integrins was observed on the endothelial cells within the tumors (Fig. 8, which is published as supporting information on the PNAS web site). This suggests that all or most endothelial cells of the tumor vessels originate from local host vessels and not from potential BM-derived endothelial cell precursors. Thus, the effects of the transplants apparently reflect circulating blood cells rather than recruitment of endothelial progenitors.

Reduced β3-Null Macrophage Infiltration in Human Tumors. Because the *Rag2*-null mice used in these experiments do not develop mature B and T lymphocytes, increased tumor growth in animals lacking β3- or β3/β5-integrins could not be attributed to an alteration in the lymphocyte population. However, it is possible that other immune cells expressing β3- or β5-integrins were not functioning properly and therefore could not block tumor growth. We tested some cells involved in the innate immune response, namely macrophages and NK cells. We analyzed the recruitment of macrophages around and within the tumors by staining the human tumor sections with an anti-F4/80 antibody (macrophage antigen) at different times. As shown in Fig. 4, macrophage infiltration can be observed in tumors grown in mice WT for integrins 3 days after injection, and fewer macrophages are present around and within the tumors grown in β3- or β3/β5-null mice. Similar results were obtained at later times (data not shown). Because of the uneven distributions of macrophages, precise quantification was difficult, but microscopic counts suggested a 6- to 10-fold reduction in the integrin-deficient mice.

Immunohistochemistry experiments also were performed for NK cells by using an anti-Pan-NK antibody. The numbers of NK cells associated with the tumors were low in all animals, and we could not see obvious differences (data not shown). To test whether reduced recruitment was due to a reduced population of monocyte/macrophage lineage or NK cells in the blood of the β3-null animals, we performed fluorescence-activated cell sorter analyses. No decrease in the F4/80-positive population or in NK cells was observed in *Rag2*/β3-null compared with *Rag2*-null/$\beta3^{+/+}$ mice (data not shown).

Tumor Growth also Is Enhanced in Selectin-Deficient Mice. Immunocompromised *Rag2*-null (control) or *Rag2*-null/P-, E-, L-, or ELP-null mice were injected s.c. with the same two human tumor cell lines. As shown in Fig. 5*a*, tumors grew in all lines of mice, but tumor size was greatly enhanced in the absence of one or all three selectins when compared with that in controls. The most significant increase was observed in the triple-selectin-knockout mice. Again, differences in growth rates were seen as early as 2 weeks after implantation and accelerated greatly after 3 weeks. Interestingly, A375 SM tumors also showed enhanced growth in

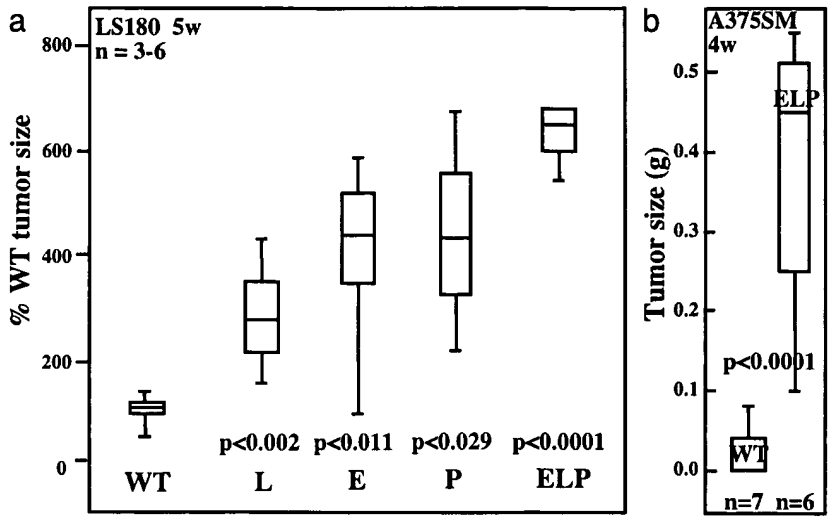

Fig. 5. Tumor growth is increased in selectin-deficient mice. (*a*) Human LS 180 s.c. tumors grown in *Rag2*-null or *Rag2*/L-, E-, P-, or ELP-null mice. (*b*) Human A375 SM s.c. tumors grown in WT or ELP-null mice. Box plots show the tumor weights as percentage of their size in selectin WT mice at 5 weeks (*a*) and in grams at 4 weeks (*b*).

triple-selectin-deficient mice (Fig. 5*b*), although these cells, unlike LS 180 cells, do not express selectin ligands (5).

Increased Tumor Growth in ELP-Null Mice Is Partially BM-Dependent. To determine whether BM-derived cells were responsible for the increased tumor growth in the ELP-null mice, we performed BM transplants as described above. Recipient *Rag2*-null or *Rag2*/ELP-null mice were lethally irradiated and reconstituted with BM derived from either *Rag2*-null or *Rag2*/ELP-null mice and then challenged with tumor cells. As before, tumor growth was reduced in all irradiated compared with nonirradiated mice (Fig. 6 *Left*; note the different scales). Tumor growth was enhanced only in mice reconstituted with BM cells derived from ELP-null mice (Fig. 6 *Center* and *Right*), suggesting the involvement of selectin-dependent, BM-derived cells in suppressing tumor growth. However, tumor growth in ELP-null mice that received a WT selectin BM transplant was slightly more pronounced than the growth in WT mice that received a WT BM transplant (compare Fig. 6 *Center* and *Right*; $P = 0.041$). This difference suggests that a BM-independent but selectin-dependent event also can affect tumor growth. Therefore, tumor growth is clearly affected not only by selectin-dependent, BM-derived cells, but also by selectins on BM-independent cells, probably endothelial cells; these express P- and E-selectins and are the only cells that express E-selectin, which clearly does have an effect (Fig. 5; see also *Discussion*).

Reduced Macrophage Infiltration in ELP-Null Mice. We analyzed the infiltration of macrophages and NK cells by staining with anti-F4/80 (macrophage antigen) or anti Pan-NK antibody at different times. As shown in Fig. 7, macrophage infiltration can be observed around and within the tumors grown in *Rag2*-null

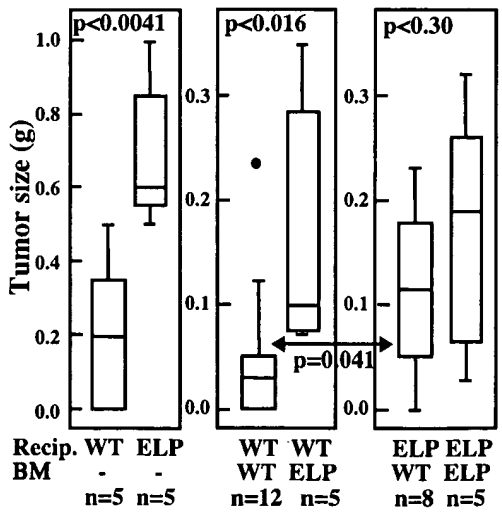

Fig. 6. Tumor growth is partially mediated by BM-derived cells in selectin-deficient mice. Human LS 180 s.c. tumors grown in mice that received BM transplants. (*Center* and *Right*) Control or selectin-null recipient (Recip.) mice were lethally irradiated and reconstituted with BM derived from either control or selectin-deficient mice and were injected s.c. 5 weeks later with LS 180 human tumor cells. (*Left*) Control mice were not irradiated and did not receive BM transplants. Box plots show the tumor weights in grams for 3-week-old tumors.

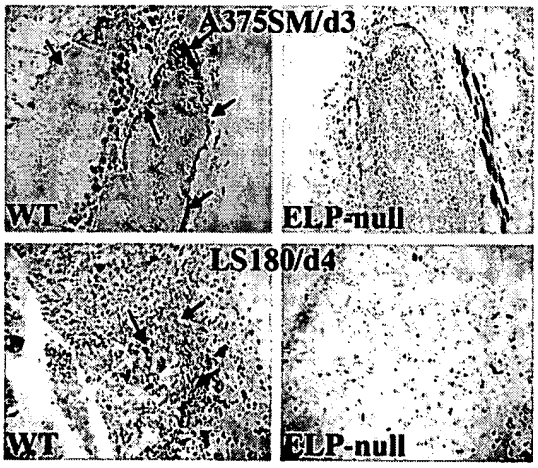

Fig. 7. Macrophage infiltration in tumors grown in *Rag2*-null or *Rag2*/ELP-null mice. Sections of 3-day-old A375 SM (*Upper*) and 4-day-old LS 180 (*Lower*) tumors were stained with an anti-macrophage antibody. A higher infiltration of macrophages (purple) around and inside the tumor was observed in tumors grown in control (*Left*) compared with selectin-deficient (*Right*) mice. Note that the skeletal muscle near the tumor is stained nonspecifically. Arrows indicate the areas occupied by macrophages. (Scale bar, 100 μm.)

mice 3 or 4 days after injection. Fewer macrophages are present in and around tumors grown in *Rag2*/ELP-null mice (a 4- to 5-fold reduction), and similar results were obtained at later times (data not shown). The numbers of NK cells were low in all sections, and we could not detect significant differences (data not shown).

Fluorescence-activated cell sorter analyses of the circulating F4/80-positive and NK cell populations showed no decreases in single, double-, or triple-selectin-null mice versus *Rag2*-null mice, in accord with previous results (15). We also checked for recruitment of BM-derived endothelial progenitors (using staining for P-selectin). As in the case of integrin-deficient mice discussed above, no recruitment was detected (data not shown). In summary, these results indicate that selectin-dependent recruitment of BM-derived circulating blood cells is important in tumor suppression.

Discussion

The results described here show clearly that certain integrins and all selectins expressed by host cells contribute to the suppression of the growth of transplanted tumors. Because β3-integrins and selectins are expressed by cells of the vessel wall (endothelial cells, pericytes, and smooth muscle cells) and by many circulating blood cells, these results could implicate numerous cell types in tumor suppression.

Prior work has suggested that αv-integrins (αvβ3 and αvβ5) could act as negative regulators of angiogenesis (1, 2, 18), and, indeed, we see enhanced vascularization of transplanted human tumors in mice lacking these integrins. The vessels appear normal, with normal investiture by pericytes, as described elsewhere for vessels lacking all αv-integrins (20). The normal association of pericytes and smooth muscle cells with the vessels suggests that failure to recruit these cells is not the reason for the enhanced angiogenesis, as has been suggested (21). An increased number of vessels could contribute to the enhanced tumor growth in integrin-deficient mice. We failed to detect any significant recruitment of BM-derived endothelial progenitors (EPCs) (22–23) to the vessels in any of the mice, although we cannot rule out a low level of recruitment or the possibility that such EPCs might require both integrins and selectins for efficient recruitment. BM-derived cells suppress, not enhance, tumor growth, whereas EPC recruitment might be expected to have an effect opposite to that which we observe. Be that as it may, our experiments clearly implicate other non-vessel wall cells derived from the BM in mediating the tumor-suppressive effects.

Given that all our experiments were conducted in *Rag2*-null mice, there was no involvement of the adaptive immune system (B, T, and NKT cells). However, many other hematopoietic cells could be involved. Many of these cells express β3-integrins and/or L-selectin and selectin ligands (also P-selectin in the case of platelets) and may use P- and E-selectins expressed by endothelial cells as well as integrins during their trafficking and extravasation (24–26). Although we certainly would not wish to rule out platelets, mast cells, eosinophils, or other minor leukocyte populations from consideration, obvious candidate cell types for involvement are neutrophils, monocytes/macrophages, and NK cells.

The numbers of circulating neutrophils in selectin-deficient mice are elevated (15) and are not significantly altered in β3-integrin-null mice. Circulating NK cells are, if anything, elevated in the integrin- and selectin-deficient mice. Histological and immunohistological analyses of the established tumors revealed few neutrophils or NK cells and no obvious differences between mice WT for or deficient in selectins or integrins. However, these results do not rule out a role for these cells, perhaps at an early time during tumor growth or acting in a "hit-and-run" fashion without significant accumulation. Extensive evidence for selectin-dependence of neutrophil traffic exists (24, 26), and some data suggest a role for αvβ3-integrin in the arrest and extravasation of these cells (27). Neutrophils can exhibit antitumor reactions (28). Recruitment of NK cells has been investigated less completely. However, it has been reported that they express the selectin ligands P-selectin glycoprotein ligand 1 (PSGL-1) and sialyl Lewis X (SLe^X) (29–30) and L-selectin (31), and selectin–ligand interactions likely contribute to their binding to the endothelium. It is well established that NK cells can kill tumor cells (32–34). We have attempted to deplete NK cells from our system, but the results were inconclusive. Such experiments, using well defined genetic backgrounds and appropriate reagents, will be required to investigate in more detail the potential involvement of NK cells and the possible roles of integrins and selectins in their targeting of tumors.

Monocytes/macrophages are known to use αvβ3-integrin and selectins in their arrest and extravasation at sites of inflammation (35). The same could be true for their infiltration of tumors, and we do detect fewer macrophages infiltrating tumors growing in integrin- or selectin-deficient mice, although the numbers of circulating monocytes/macrophages do not differ among the strains of mice (15). Macrophages have been suggested to be tumoricidal (36–39), although other reports have suggested that they also can contribute to tumor growth (40–42); they probably have both effects (42–46).

To dissect further the possible roles of NK cells and macrophages (and other hematopoietic cells) in the adhesion receptor-dependent tumor suppression that we report here, it will be necessary to perform lineage-specific reconstitution and ablation (genetic or pharmacological). Those experiments should shed light on the role of innate immunity against tumors and the roles of specific adhesion receptors in mediating their effects.

We thank Rakesh Jain and Denisa Wagner for critically reading the manuscript; Melanie Yanek and Matthew Fogarty for technical assistance; and Rod Bronson for help interpreting the histology. This work was supported by National Institutes of Health Grants RO1CA17007 (to R.O.H) and RO1CA38701 (to A.V.) and by the Howard Hughes Medical Institute. R.O.H. is an Investigator and D.T. was an Associate of the Howard Hughes Medical Institute.

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[CANCER RESEARCH 62, 2824–2833, May 15, 2002]

Multiple Roles for Platelet GPIIb/IIIa and αvβ3 Integrins in Tumor Growth, Angiogenesis, and Metastasis

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ABSTRACT

In vivo **tumor cells interact with a variety of host cells such as endothelial cells and platelets, and these interactions are mediated by integrins GPIIb/IIIa and αvβ3. We used chimeric (c) 7E3 Fab (ReoPro) and murine (m) 7E3 F(ab')$_2$ to elucidate the role of these integrins in angiogenesis, tumor growth, and metastasis. These antibodies are potent inhibitors of GPIIb/IIIa and αvβ3. c7E3 Fab inhibited αvβ3-mediated human umbilical vein endothelial (HUVEC) and melanoma cell adhesion, migration, invasion, and basic fibroblast growth factor stimulated proliferation of HUVECs (IC_{50} values range from 0.15 to 5 μg/ml for different assays). In an *in vitro* angiogenesis assay, c7E3 Fab inhibited basic fibroblast growth factor and platelet-stimulated capillary formation of HUVECs (IC_{50} = 10 μg/ml and 15 μg/ml, respectively), demonstrating that endothelial αvβ3 is important for sprouting, and platelet-stimulated sprouting is mediated by GPIIb/IIIa. In an experimental metastasis assay, a single pretreatment of human melanoma cells with c7E3 Fab (2.5 μg/ml) inhibited lung colonization of the tumor cells in severe combined immunodeficient mice. *In vivo*, m7E3 F(ab')$_2$ partially inhibited growth of human melanoma tumors in nude mice compared with control-treated animals. These data suggest that tumor cell-expressed integrins are important but not the only component involved in tumor growth. Because c7E3 Fab and m7E3 F(ab')$_2$ do not cross-react with murine integrins, this inhibition of metastasis and tumor growth is attributable to direct blockade of human tumor αvβ3 integrins. m7E3 F(ab')$_2$ completely blocked tumor formation and growth of human melanoma tumors growing in nude rats. In this xenograft model, m7E3 F(ab')$_2$ simultaneously binds to both human tumor and host platelet GPIIb/IIIa and endothelial αvβ3 integrins, thus participating as an antiangiogenic and an antitumor agent. Collectively, these results indicate that combined blockade of GPIIb/IIIa and αvβ3 affords significant antiangiogenic and antitumor benefit.**

INTRODUCTION

c7E3[2] Fab (abciximab; ReoPro) is a mouse-human chimeric mAb Fab fragment of the parent murine mAb 7E3. c7E3 Fab was the first agent to be approved for use as adjunct therapy for the prevention of cardiac ischemic complications in patients undergoing percutaneous coronary intervention (1). c7E3 Fab binds with high avidity to the GPIIb/IIIa (also known as αIIbβ3) receptor on platelets, which is the major receptor involved in platelet aggregation. c7E3 Fab also binds with equivalent affinity to the vitronectin receptor αvβ3, and it can redistribute between GPIIb/IIIa and αvβ3 receptors *in vitro* (2). We asked whether c7E3 Fab could be used to determine the contribution of platelet GPIIb/IIIa and αvβ3 integrins in tumor growth, angiogenesis, and metastasis.

There is now considerable evidence that progressive tumor growth is dependent on angiogenesis. The formation of new blood vessels provides tumors with nutrients and oxygen, allows the removal of waste products, and acts as conduits for the spread of tumor cells to distant sites (3). Several studies have defined the role of integrins in the angiogenic process (4–6). During the angiogenic process, αvβ3 is up-regulated on the surface of activated endothelial cells, which in turn helps these cells to migrate, proliferate, and invade the tumor (4–6). An antagonist of αvβ3, LM609, suppressed angiogenesis and blocked growth of human tumors that did not express this receptor (7). LM609 was used in a SCID mouse human chimeric angiogenesis model. In this system, αvβ3-negative human melanoma cells were injected into full thickness human skin grafted onto SCID mice. The resulting tumors induced an angiogenic response that enhanced the growth of tumor cells in an orthotopic microenvironment (7). Regular administration of LM609 significantly inhibited growth of αvβ3-negative tumors by blocking the growth of human blood vessels. Because LM609 does not cross-react with mouse integrins, its antiangiogenic activity was attributed to blockade of human αvβ3 receptors in the vasculature of the human skin. A subsequent study using the murine IgG equivalent of c7E3 Fab (m7E3 IgG) in the same model achieved similar results as LM609 (8). Similar to LM609, 7E3 does not cross-react with mouse integrins; therefore, it inhibited growth of human tumors by blocking human αvβ3 receptors in the vasculature of the human skin. In these studies, a partial inhibition of tumor growth was observed, and the combined effect of blocking tumor cell-expressed αvβ3 and endothelial cell-expressed αvβ3 was not evaluated. One limitation of this model is that tumors can grow even in the absence of human vasculature, because the mouse vasculature can sustain tumor growth. To the best of our knowledge, a relevant model examining simultaneous blockade of both host and tumor cell-expressed integrin has not yet been evaluated. One purpose of our study was to evaluate whether combined blockade of host and tumor cell-expressed integrins was superior to blockade of tumor cell-expressed integrins *in vivo*.

The clinical significance of β3 integrin expression in human melanoma was determined in a prospective study that examined the expression of this integrin in patients who were followed for a mean of 98 months after diagnosis with intermediate thickness melanoma (9, 10). This study concluded that tumors in 64% of the patients expressed β3 integrin, and a higher proportion (45%) of patients with β3 positive melanomas were more likely to die of their disease when compared with those with β3 negative tumors (8%).

Angiogenesis can also stimulate the metastatic cascade by providing conduits for the spread of tumor cells to distant sites (6, 11). Some have postulated that platelets are involved in tumor cell extravasation, adherence, or trapping of tumor cell-platelet aggregates to capillary walls, and protection of circulating tumor cells from the antitumor response of the host (reviewed in Refs. 1, 11, 12). The role of platelets in facilitating hematogenous metastasis is well accepted, but little is known about their role in contributing to growth of the primary and/or metastatic tumor. Platelet granules contain a variety of angiogenic factors such as VEGF, platelet-derived growth factor, TGF-β, and fibrinogen, and these modulators are immediately secreted after platelet activation (13). Tumor vasculature is leaky, and extravasated

Received 11/1/01; accepted 3/18/02.

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[2] The abbreviations used are: c, chimeric; m, murine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; mAb, monoclonal antibody; HUVEC, human umbilical vein endothelial cell; bFGF, bovine basic fibroblast growth factor; MC, microcarrier; PRP, platelet-rich plasma; PPP, platelet-poor plasma; 3x/wk, three times per week; 5x/week, five times per week; SCID, severe combined immuno-deficient.

fibrin(ogen) that is deposited on the tumor surface can provide an ideal substrate for platelet binding and activation. In addition, tumor cells can activate platelet aggregation (14) and cause the release of VEGF from platelets (15, 16), which in turn can stimulate angiogenesis. c7E3 Fab can block GPIIb/IIIa-mediated platelet aggregation, degranulation, and adhesion to fibrinogen (1). One goal of this study was to determine whether blockade of platelets could inhibit tumor growth *in vivo*. Recently, Verheul *et al.* (17) have demonstrated that platelets stimulate endothelial cell proliferation *in vitro*. Clinically thrombocytosis, an increase in platelet count, is directly correlated with survival of patients of lung and ovarian carcinoma (18–20), supporting the notion that platelets may play a role in tumor growth, angiogenesis, and metastasis. The central hypothesis for our study was that combined blockade of platelet GPIIb/IIIa, endothelial, and tumor cell-expressed αvβ3 could have an enhanced inhibitory effect compared with blockade of tumor cell-expressed αvβ3 alone. c7E3 Fab is one such agent that can antagonize GPIIb/IIIa and αvβ3, and it is widely used in the clinic as an antithrombotic agent. Therefore, we wanted to determine whether c7E3 Fab has anticancer properties. Results from this study indicate that c7E3 Fab and m7E3 $F(ab')_2$, in addition to providing antithrombotic effect, also possess antiangiogenic and antitumor properties.

MATERIALS AND METHODS

Reagents. Bovine bFGF and human $VEGF_{165}$ were obtained from R&D Systems (Minneapolis, MN). mAb 1976Z (LM609), a mAb against integrin αvβ3, and MAB1961 (PIF6), a mAb against integrin αvβ5, were purchased from Chemicon (Temecula, CA). Biocoat cell culture inserts (pore size 8 μm) were purchased from Becton Dickinson (Bedford, MA). Vybrant cell adhesion assay kit (V-13181) was purchased from Molecular Probes (Eugene, OR). Human plasminogen-free fibrinogen (von Willebrand/fibronectin depleted) was purchased from Enzyme Research Labs (South Bend, IN). Bovine skin gelatin was purchased from Sigma (St. Louis, MO). Human vitronectin was purchased from Promega (Madison, WI), and type I collagen from Life Technologies, Inc. (Gaithersburg, MD). c7E3 Fab, m7E3 $F(ab')_2$, and 10E5 were generated at Centocor. For animal experiments m7E3 $F(ab')_2$, instead of the intact IgG, was used to eliminate platelet clearance and any other Fc receptor-mediated events.

Cell Lines. HUVECs were purchased from Clonetics (Walkersville, MA), and cultured in EBM complete medium (Clonetics) containing 10% fetal bovine serum, long R insulin-like growth factor-1, ascorbic acid, hydrocortisone, human epidermal growth factor, human VEGF, gentamicin sulfate, and amphotericin-B. Cells were grown at 37°C and 5% CO_2, and medium was changed every 2–3 days. Cells were passaged when they reached 80% confluence. Passages 3–8 were used in all of the experiments. The A375S2 human melanoma cell line was obtained from American Type Culture Collection (Rockville, MD), and deemed free of *Mycoplasma* and bacterial contaminants. The cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. HT168M1 melanoma cells were isolated from a patient as described (21) and were cultured in 10% FBS and RPMI 1640. Human colon carcinoma HT29 cells were obtained from American Type Culture Collection, and deemed free of *Mycoplasma* and bacterial contaminants. The cells were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, and 0.1 mM nonessential amino acids.

Flow Cytometry. To stain surface integrins, cells were harvested, rinsed, suspended in unsupplemented RPMI 1640, and sequentially incubated for 60 min at room temperature with anti-integrin mAbs (10 μg/ml) and FITC-labeled goat antimouse antibody (1:200). In some instances, cells were directly labeled with FITC-labeled anti-integrin mAbs (10 μg/ml). Absence of primary antibody or substitution of primary antibody with isotype-matched irrelevant antibody served as negative controls. Cells were immediately analyzed with a fluorescence-activated cell sorter Scan II flow cytometer (Becton Dickinson, Mountain View, CA).

Adhesion Assay. Microtiter plates (Linbro-Titertek; ICN Biomedicals, Inc.) were coated at 4°C overnight with vitronectin (1 μg/ml), gelatin (0.1%), fibrinogen (100 μg/ml), type I collagen (10 μg/ml), or fibronectin (10 μg/ml). Fibrin-coated Microtiter wells were formed by thrombin treatment (1 units/ml) of fibrinogen. These concentrations of proteins supported optimal cell adhesion. Immediately before use plates were rinsed with PBS and blocked for 1 h with 1% BSA/PBS (pH 7.4). Adherent cells were labeled with Calcein a.m. fluorescent dye (Molecular Probes) according to the manufacturer's instructions, harvested, washed twice, and suspended in 0.1% BSA in DMEM. After cell density was adjusted to 5×10^5/ml, cells were incubated with various concentrations of antibodies for 15 min at 37°C. The cell-antibody mixture was

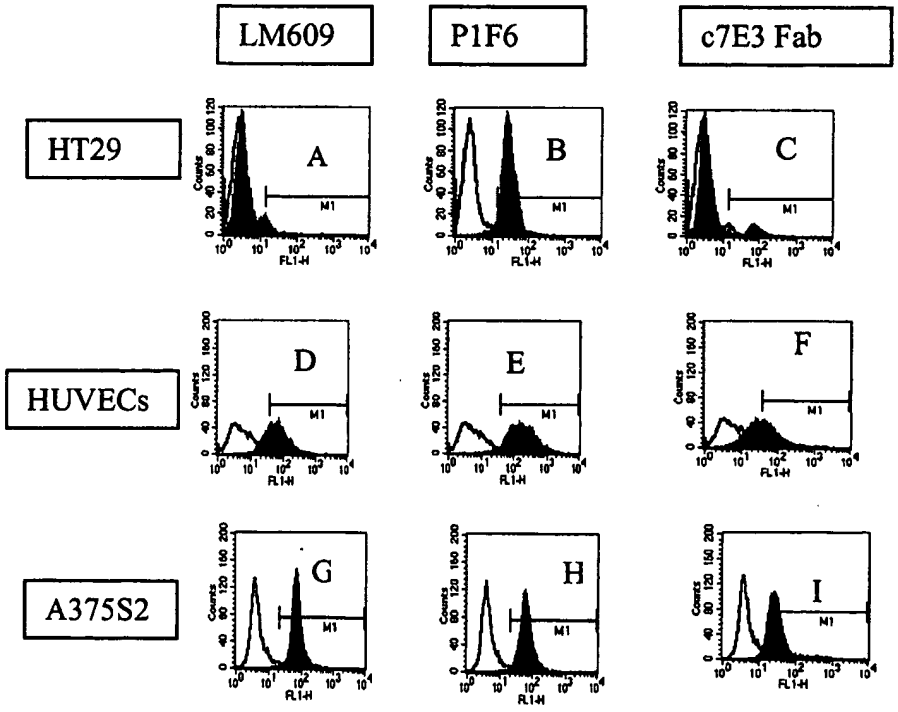

Fig. 1. HT29 cells (*A–C*) express αvβ5 but not αvβ3 integrin on their surface. HUVEC (*D–F*) and A375S2 (*G–I*) cells express αvβ5 and αvβ3 integrin on their surface. Tumor cells and endothelial cells were stained by immunofluorescence and analyzed by flow cytometry. The *histogram on the left* represents background fluorescence in the presence of isotype matched antibody. The *histogram on the right* indicates staining of test antibody. *A, D,* and *G,* LM609 (mAb directed to αvβ3, 10 μg/ml); *B, E,* and *H,* PIF6 (mAb directed to αvβ5, 10 μg/ml); and *C, F,* and *I,* c7E3 Fab (10 μg/ml). *M1,* marker that indicates the gate for positive cells.

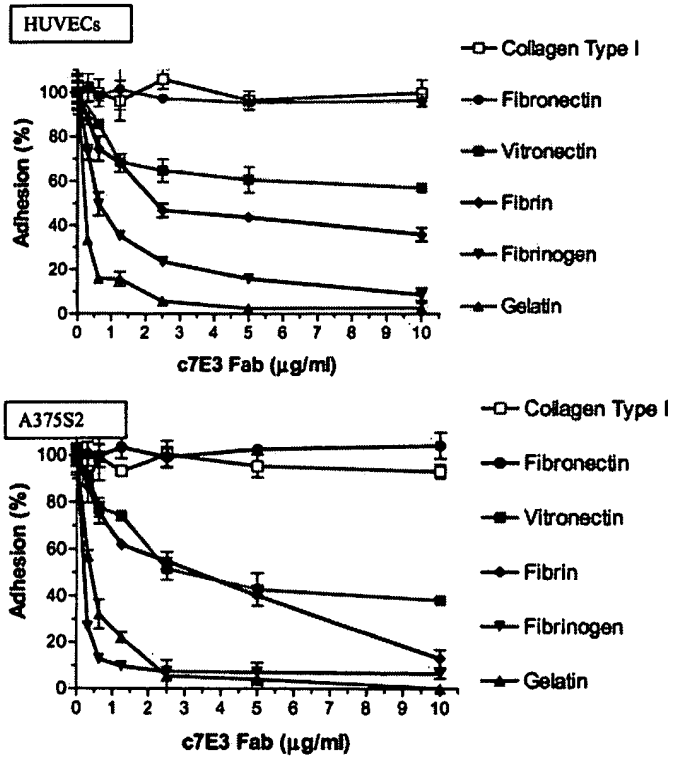

Fig. 2. Adhesion of HUVECs and A375S2 melanoma cells to matrix proteins. Adhesion assay was performed as described in "Materials and Methods." Cell adhesion to BSA-coated wells served as a negative control. Extent of cell adhesion in the presence of various concentrations of antibody was plotted as a percentage of cell adhesion in the absence of antibody that was considered as 100%. Each data point is the mean of triplicate determinations and is representative of at least three experiments; *bars*, ± SD.

added to wells (100 μl/well) and incubated for 1 h at 37°C. Plates were rinsed twice with PBS to remove unbound cells, and adhesion was measured in a fluorescence plate reader (Fluoroskan; Tecan, Research Triangle Park, NC) at 485–538 nm. Cell adhesion to BSA-coated wells served as a negative control. Isotype-matched antibodies served as a negative control.

Cell Migration Assay. Cell migration assays were performed in 24-transwell chambers with a polystyrene membrane (6.5-mm diameter, 10-μm thickness, and 8-μm pore size) as described previously (22). Briefly, the underside of the membrane was coated with vitronectin (2 μg/ml) for 60 min at room temperature and then blocked with a solution of 1% BSA/PBS at room temperature for 60 min. Next, membranes were washed with PBS and dried. Serum-free medium (750 μl) containing 0.1% BSA and bFGF (20 ng/ml) or medium containing 10% FBS was added to the lower chambers. Subconfluent 24-h cultures were harvested with trypsin-EDTA, washed twice, and resuspended in serum-free medium. Cells (100,000/500 μl) were added to the upper chambers in the presence or absence of antibodies. The chambers were placed in a tissue culture incubator, and migration was allowed to proceed for 4–6 h. Migration was terminated by removing the cells on the top with a cotton swab, and the filters were fixed with 3% paraformaldehyde and stained with Crystal Violet. The extent of cell migration was determined by light microscopy, and images were analyzed using the Phase 3 image analysis software (Glen Mills, PA). The software analyzes the total area occupied by the stained cells on the bottom side of the filter, and this is directly proportional to the extent of cell migration.

Invasion Assay. The cell invasion assays were performed as described (23). Briefly, fibrinogen (plasminogen-free 100 μl of 10 mg/ml) and 100 μl of 1 unit/ml thrombin was mixed, and immediately added to the top chamber of 24-well transwell plates (6.5-mm diameter, 10-μm thickness, and 8-μm pore size). The plates were incubated at 37°C for 30 min to form a fibrin gel. Confluent tumor cells (A375S2) were trypsinized, centrifuged, resuspended in basal medium supplemented with 0.1% BSA and 10 μg/ml plasminogen (Enzyme Research Labs) with various concentrations of antibodies, and incubated for 15 min at room temperature. Cells (100,000/500 μl) were added to the upper chamber in the presence or absence of antibodies. The lower compartment of the invasion chamber was filled with 0.75 ml of 10% FBS-DMEM, which served as a chemoattractant, and the plate was transferred to a tissue culture incubator. After 24 h, invasion was terminated by removing the cells on the top with a cotton swab, and the filters were fixed with 3% paraformaldehyde and stained with Crystal Violet. The extent of cell migration was analyzed using the Phase 3 image analysis software as described above.

Endothelial MC-based Sprouting Assay. A modification of the methods of Nehls and Drenckhahn (24) was used to measure capillary tube formation in three-dimensional fibrin-based matrix. Gelatin-coated cytodex-3 MCs (Sigma) were prepared according to recommendations of the supplier. Freshly autoclaved MCs were suspended in EBM-2 + 20% FBS, and endothelial cells were added to a final concentration of 40 cells/MC. The cells were allowed to attach to the MCs during a 4-h incubation at 37°C. The MCs were then suspended in a large volume of medium and cultured for 2–4 days at 37°C in 5% CO_2 atmosphere. MCs were occasionally agitated to prevent aggregation of cell coated beads. MCs were embedded in a fibrin gel that was prepared as follows: human fibrinogen (2 mg/ml) was dissolved in plain medium containing antibodies and/or bFGF, PRP containing 250,000 platelets/μl, PPP, or serum containing EBM-2 medium. PRP, PPP, and gel-filtered platelets were prepared from citrated whole blood obtained from healthy volunteers as described (2). To prevent excess fibrinolysis by fibrin-embedded cells, aprotinin was added to the fibrinogen solution and to growth medium at 200 units/ml. Cell-coated MCs were added to the fibrinogen solution at a density of 100–200 MCs/ml (50–100 beads/per 48-well plate), and clotting was induced by addition of thrombin (0.5 units/ml). After clotting was complete, 0.5 ml of solution (containing all of the components described above except fibrinogen and thrombin) was added to the fibrin matrices. The plates were incubated at 37°C

Table 1 *Adhesion of HUVECs and A375S2 to vitronectin, gelatin, fibrinogen, fibrin, fibronectin, and type I collagen*

Extent of cell adhesion in the presence of various concentration of antibody was plotted as a percentage of cell adhesion in the absence of antibody that was considered as 100%. Each data point is the mean of triplicate determinations (±SD). The concentration of antibodies used was 10 μg/ml.

A. Adhesion of HUVECs (%) ±SD

	Vitronectin	Gelatin	Fibrinogen	Fibrin	Fibronectin	Type I collagen
Human IgG	96.3 ± 11.4	109.0 ± 8.8	108.0 ± 6.3	99.7 ± 4.5	96.8 ± 4.7	99.3 ± 4.1
LM609	26.3 ± 3.7	36.5 ± 4.7	14.3 ± 2.5	48.1 ± 1.5	102.8 ± 7.2	108.8 ± 12.7
PIF6	39.8 ± 5.9	94.4 ± 15.1	94.5 ± 4.2	96.7 ± 4.5	103.2 ± 3.8	115.7 ± 8.1
LM609 + PIF6	3.7 ± 0.4	32.2 ± 5.2	10.7 ± 1.1	30.7 ± 8.9	99.6 ± 4.7	116.2 ± 4.1
c7E3 Fab	54.9 ± 0.9	2.5 ± 2.3	8.7 ± 2.9	35.8 ± 3.0	96.3 ± 2.8	99.6 ± 6.0

B. Adhesion of A375S2 (%) ±SD

	Vitronectin	Gelatin	Fibrinogen	Fibrin	Fibronectin	Type I collagen
Human IgG	104.0 ± 5.3	94.6 ± 12.4	102.5 ± 5.9	99.5 ± 4.0	100.0 ± 5.5	99.1 ± 3.3
LM609	42.1 ± 6.1	25.2 ± 7.1	14.0 ± 1.8	50.0 ± 1.9	104.0 ± 8.1	100.0 ± 1.5
PIF6	28.5 ± 3.8	87.4 ± 7.8	99.4 ± 3.6	92.9 ± 4.7	101.0 ± 5.7	101.0 ± 7.3
LM609 + PIF6	0.9 ± 0.3	1.1 ± 1.5	10.3 ± 2.6	47.6 ± 3.2	109.0 ± 4.1	102.0 ± 4.6
c7E3 Fab	38.1 ± 0.7	6.0 ± 1.0	6.5 ± 2.1	12.9 ± 3.8	104.0 ± 5.6	93.1 ± 3.1

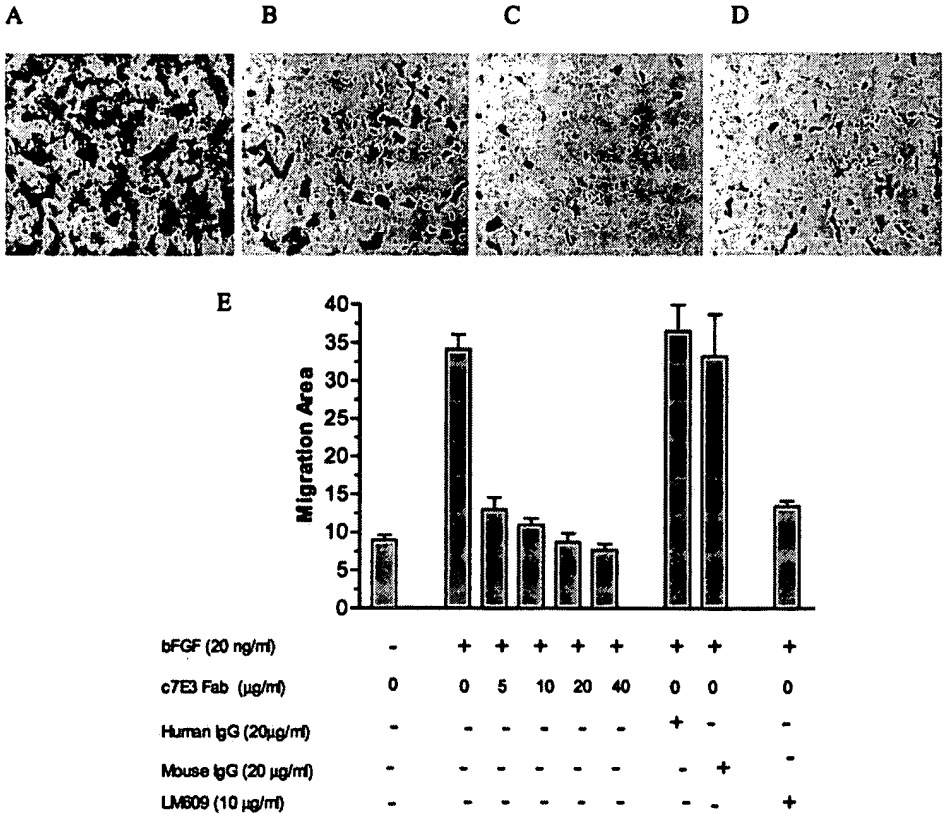

Fig. 3. Migration of HUVECs toward vitronectin in the presence of bFGF. The undersides of migration chamber filters were coated with 2 μg/ml of vitronectin, and the assay was performed as described in "Materials and Methods." Cells were allowed to migrate for 6 h. Each data point is the mean of 3 transwell filters; *bars*, ± SD. Digital photomicrographs of endothelial cell migration in the presence of *A*, bFGF (20 ng/ml) + control antibody (20 μg/ml); *B*, bFGF (20 ng/ml) + c7E3 Fab (5 μg/ml); *C*, bFGF (20 ng/ml) + c7E3 Fab (40 μg/ml); and *D*, control antibody (20 μg/ml) and absence of bFGF. *E*, graphical representation of inhibition of cell migration in the presence of various antibodies.

and 5% CO_2 for 1–3 days. After 1–3 days, gels were fixed with a solution of 3% paraformaldehyde in PBS, and the number of capillary sprouts with length exceeding the diameter of the MC bead (150 μm) was quantified by using the Phase 3 image analysis.

Endothelial Cell Proliferation and Apoptosis Assays. Subconfluent HUVECs were trypsinized, washed, and resuspended in complete medium. Cells (5000) were added to each well of 96-well plates. To test whether the plates themselves may influence the assay, endothelial cells were plated on normal tissue culture plates, high protein-binding plates that were precoated with vitronectin (1 μg/ml), gelatin (0.1%), or type I collagen (2 μg/ml). Cells were allowed to attach for 2 h, medium was aspirated, wells were washed once with PBS, and 100 μl of medium (0.1% serum-M199 or 2% serum-M199) containing bovine bFGF-2 (R&D systems), human recombinant $VEGF_{165}$ (r + D Systems), and/or various antibodies was added to each well. The plates were incubated at 37°C for 48 h. Extent of cell proliferation was determined by the Celltiter 96 Aqueous kit (Promega), ATP kit (Packard, Meridian, CT), or BrdUrd kit (Oncogene Research Products). For the MTS and the BrdUrd assay, absorbance was measured at 490 nm and 540/450 nm, respectively. Luminescence intensity was measured for the ATP assay in a TopCount reader (Packard). To quantify apoptosis, cells were treated as above with antibodies or positive control etoposide for 18 h, and extent of DNA fragments were measured by using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany).

Matrigel-based Angiogenesis Assay in Nude Rats. The Matrigel plug-based angiogenesis assay was performed as described earlier (25) with slight modifications. Briefly, cold Matrigel (Becton Dickinson) was mixed with bFGF (5 μg/ml) and m7E3 $F(ab')_2$ (~300 μg/ml) or an equal volume of PBS. The next day, 2 ml of Matrigel solution was injected s.c. into nude rats (Taconic, Germantown, NY), and animals were dosed i.p. with 6 mg/kg of m7E3 $F(ab')_2$. This dose of m7E $F(ab')_2$ completely inhibits rat platelet aggregation *ex vivo* (26). Animals were dosed every day for 6 days, and plugs were removed on day 7. The extent of angiogenesis was quantified by using the Drabkins kit (Sigma) as described (25).

Lung Metastasis Assay. The lung metastasis assay was performed as described previously (27). Human melanoma HT168M1 cells were pretreated with 2.5 μg/ml of c7E3 Fab or control antibody for 15 min at room temperature, washed, and 1 × 10^6 cells were tail vein injected into female SCID mice. After 1 month, animals were euthanized, lungs were removed and fixed in paraformaldehyde, and the number of lung colonies were counted.

Growth of Human Melanoma Tumors in Nude Mice and Nude Rats. To determine whether m7E3 $F(ab')_2$ could inhibit tumor growth *in vivo*, we used a human melanoma xenograft model in nude mice and nude rats. Briefly, A375S2 cells (3 × 10^6/animal) were s.c. injected into female nude mice (Charles River, Raleigh, NC) or nude rats (Taconic). Tumor cells were pretreated with antibody (100 μg/ml for 5 min) before injection or therapy was initiated after animals had developed measurable tumors. Antibody was injected i.p. at a dose of 200 μg/animal or at an animal body weight-adjusted dose of 3–10 mg/kg. Control groups were injected with equivalent volume of diluent (PBS). Tumor volume (mm^3) was calculated based on the formula: (length × width × width)/2 and tumor wet weight (mg) was obtained at termination of the study.

RESULTS

Endothelial and Tumor Cell Adhesion to Matrix Proteins. Flow cytometry was used to characterize integrin expression. A375S2 and HUVECs expressed both αvβ3 and αvβ5 integrins, whereas HT29 cells expressed αvβ5 but not αvβ3 (Fig. 1). A375S2 and HUVECs but not HT29 cells stained with c7E3 Fab (Fig. 1). Therefore, we used A375S2 and HUVECs to determine the effects of αvβ3 blockade in tumor growth and angiogenesis.

Because αvβ3 binds gelatin, fibrinogen, fibrin, and vitronectin (28, 29), we questioned whether c7E3 Fab could block cell adhesion to these matrix proteins. c7E3 Fab completely inhibited adhesion of HUVECs and A375S2 cells to fibrinogen and gelatin, and it partially inhibited cell adhesion to vitronectin (Fig. 2*A*; Table 1). c7E3 Fab completely inhibited tumor cell adhesion to fibrin, whereas it partially blocked endothelial cell adhesion to fibrin (Fig. 2; Table 1), suggesting that endothelial cells use more than the αvβ3 receptor to adhere to fibrin. Because HT-29 cells do not express αvβ3-integrin, c7E3

Fab did not block cell adhesion (data not shown). Collectively, the data indicate that αvβ3 mediates cell adhesion (Figs. 2 and 3; Table 1).

Migration of Human Melanoma and Endothelial Cells. Results described above indicate that c7E3 Fab blocked αvβ3-mediated cell adhesion, therefore we determined whether c7E3 Fab could block cell migration. c7E3 Fab dose dependently inhibited bFGF-stimulated endothelial cell migration (Figure 3). Interestingly, c7E3 Fab also inhibited migration of A375S2 when serum was used as a chemoattractant (Figure 4). Although c7E3 Fab only partially inhibited cell adhesion to vitronectin (Figure 2), it completely blocked cell migration towards this matrix protein. Similar results were obtained with LM609, P1F6 and the combination of both antibodies (Table 1). 10E5 did not block migration of A375S2 cells, suggesting that GPIIb/IIIa is not functionally expressed in this tumor cell line. These findings suggest that endothelial and melanoma cells primarily use αvβ3 integrin to migrate towards vitronectin, and c7E3 Fab can inhibit both bFGF and serum stimulated cell migration.

Human Melanoma Cell Invasion through Fibrin. Because c7E3 Fab inhibited cell adhesion and migration, we determined whether this antibody could block invasion of tumor cells. Invasion is a multistep process that involves cell adhesion, degradation of the matrix, and migration of cells through the degraded matrix. We chose fibrin as a matrix for tumor cells because peritumoral deposition of fibrin *in vivo* facilitates tumor cell extravasation and hematogeneous spread (30). Invasion of A375S2 cells was inhibited by LM609 (Fig. 5), suggesting the involvement of at least αvβ3 in this process. Similarly, c7E3 Fab dose-dependently inhibited tumor cell invasion through fibrin. P1F6 was only partially effective at inhibiting tumor cell invasion, and no enhanced inhibition was observed when it was combined with LM609, suggesting that αvβ5 is involved to a lesser degree than αvβ3 in tumor cell invasion. Irrelevant IgG and a mAb directed to platelet GPIIb/IIIa (10E5) served as negative controls. Collectively, these data suggest that blockade of αvβ3 by c7E3 Fab can effectively block invasion of human melanoma cells.

c7E3 Fab Inhibits Proliferation of Endothelial Cells by Promoting Apoptosis. Because angiogenesis involves not only endothelial cell adhesion, migration, and invasion, but also endothelial cell proliferation, we asked whether c7E3 Fab could inhibit proliferation of endothelial cells. c7E3 Fab dose-dependently blocked endothelial cell proliferation stimulated by bFGF and serum (Fig. 6). c7E3 Fab inhibited cell proliferation by inducing apoptosis of proliferating endothelial cells (Fig. 6*B*). No effect of the drug was observed on quiescent endothelial cells, suggesting that αvβ3 function is only essential for proliferating endothelial cells. These findings indicate that c7E3 Fab inhibits endothelial cell proliferation in response to serum and bFGF, suggesting that αvβ3 plays a central role in mediating endothelial cell proliferation.

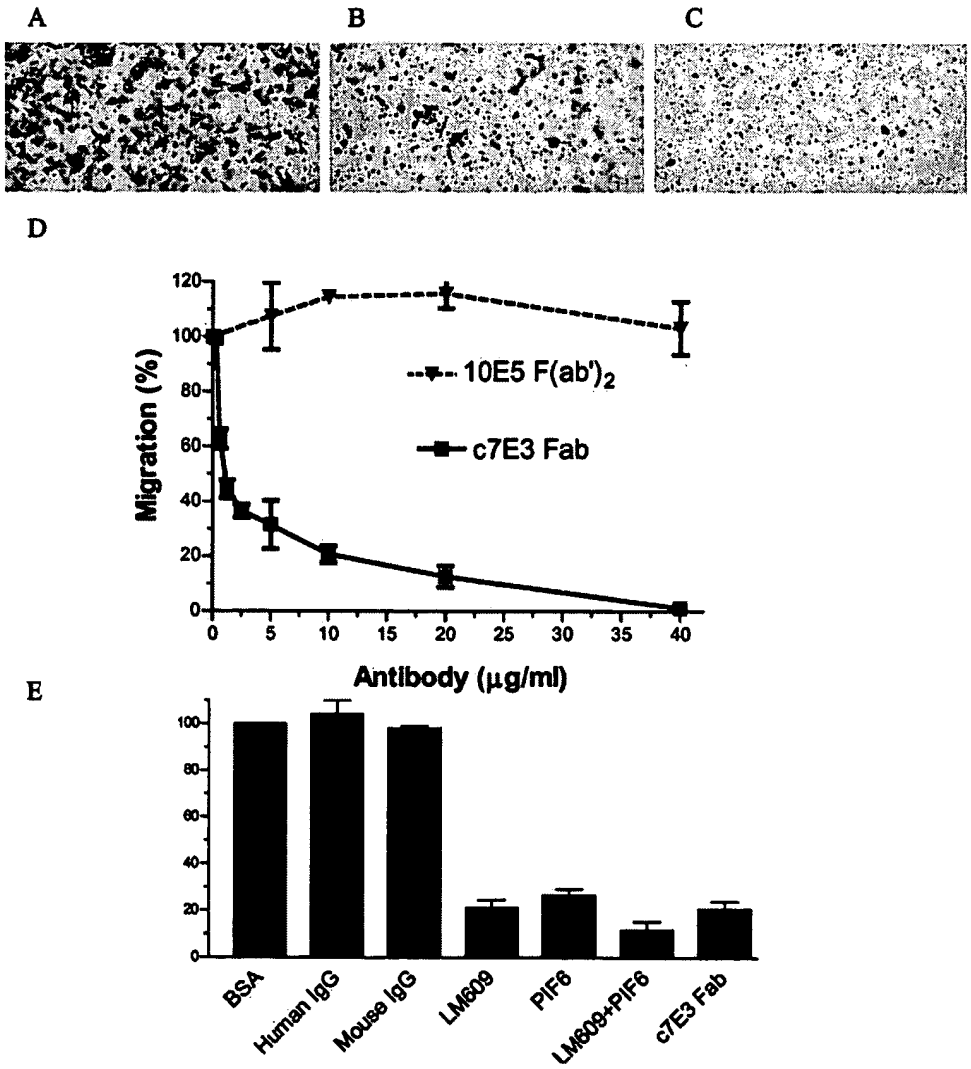

Fig. 4. Migration of A375S2 cells toward 10% FBS. Migration assay was allowed to proceed for 4 h, and the data were captured as described in "Materials and Methods." Digital photomicrographs of tumor cell migration in: (*A*) absence, or presence of c7E3 Fab (*B*) 5 μg/ml and (*C*) 20 μg/ml. *D*, graphical representation of cell migration in the presence of various concentrations of c7E3 Fab or 10E5 F(ab')$_2$. *E*, graphical representation of cell migration in the presence of 10 μg/ml of various antibodies or BSA. The data were normalized to percentage of control (BSA), which was considered as 100%, and each point is the mean of three transwell filters; *bars*, ± SD.

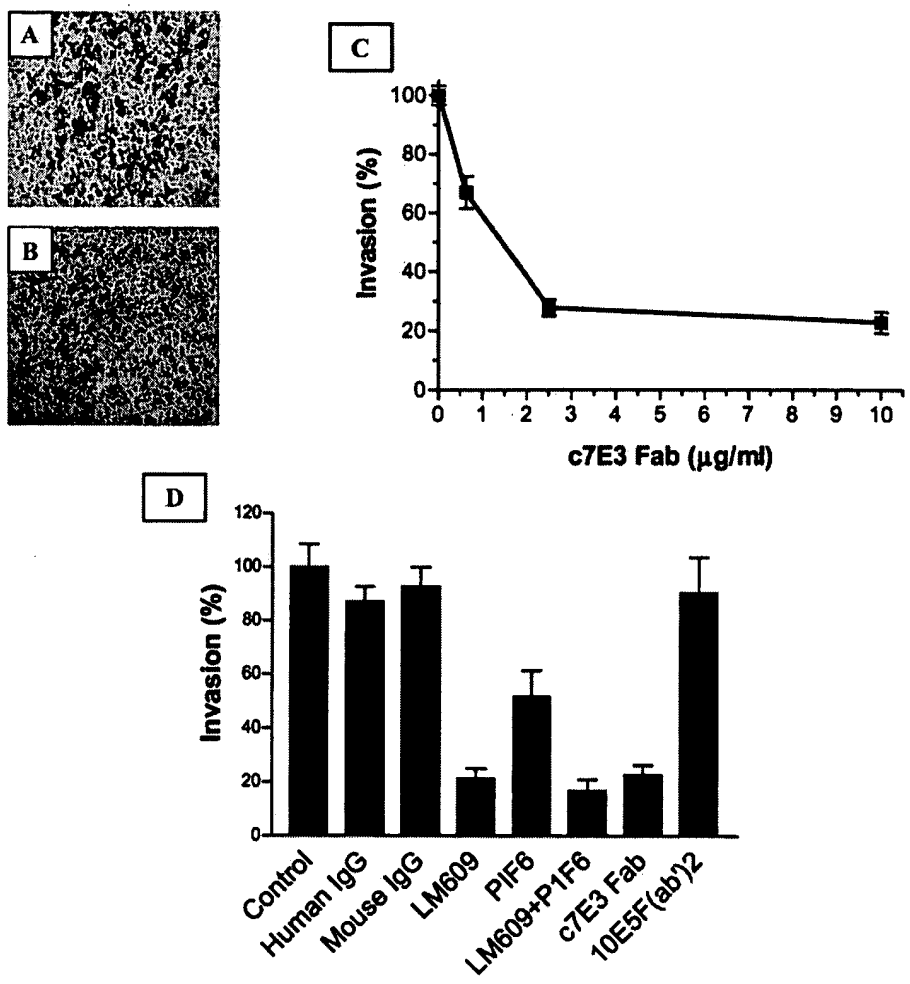

Fig. 5. Invasion of A375S2 cells through a fibrin gel. Invasion was allowed to proceed for 24 h, and the assay was performed as described in "Materials and Methods." Photomicrographs are representative fields (×4 objective lens) of cell invasion in (*A*) the absence of antibodies or (*B*) c7E3 Fab (10 μg/ml). *C*, dose-dependent inhibition of tumor cell invasion by c7E3 Fab. *D*, invasion in presence of various antibodies at (10 μg/ml). The data were normalized to percentage of control (no antibody), which was considered as 100%, and each point is the mean of three transwell filters and the graphs are representative of at least three separate experiments; *bars*, ± SD.

Inhibition of Endothelial Cell Sprouting. Endothelial cell sprouting in a three-dimensional fibrin gel is highly representative of angiogenesis. This assay involves endothelial cell proliferation, adhesion, spreading, migration, and invasion of endothelial cells. To confirm and extend the findings described above, we determined whether c7E3 Fab could block experimental angiogenesis in this assay. c7E3 Fab dose-dependently blocked bFGF stimulated endothelial sprouting in a fibrin gel (Fig. 7*B*). Isotype-matched irrelevant mouse and human antibodies served as negative controls.

In addition to bFGF, several other factors such as VEGF and TGF-β can stimulate endothelial cell proliferation. Platelet granules contain many growth factors including VEGF (13) that are secreted on platelet activation and aggregation. c7E3 Fab blocks VEGF release from platelets *in vitro* (15, 16). Therefore, we explored whether platelets could stimulate endothelial cell sprouting and whether c7E3 Fab could block this effect. Fig. 7*C* indicates that PRP stimulated endothelial sprouting to a greater extent than PPP, suggesting that platelets can stimulate angiogenesis. c7E3 Fab completely inhibited PRP-stimulated endothelial cell sprouting; however, because c7E3 Fab also blocks αvβ3, it was difficult to interpret whether platelet GPIIb/IIIa was involved in platelet-stimulated endothelial sprouting. The involvement of GPIIb/IIIa was demonstrated by the observation that 10E5, a mAb to GPIIb/IIIa, completely blocked gel-filtered platelet-stimulated endothelial cell sprouting (Fig. 7*D*). Inhibition of both endothelial αvβ3 and platelet GPIIb/IIIa receptor inhibited endothelial sprouting that was stimulated by either platelets or by angiogenic factors contained in plasma such as bFGF.

c7E3 Fab Inhibits Experimental Metastasis of Human Melanoma Tumors. Earlier studies indicated that m7E3 F(ab')$_2$ recognizes rat integrins but not murine integrins, and it blocks experimental metastasis of mouse tumor cells in a rat (15). The proposed antimetastatic mechanism that explains these results is that the antibody blocks the host (rat) platelet GPIIb/IIIa and αvβ3 integrins, thereby preventing seeding of the murine tumor cells in the lung endothelium. To test if blockade of tumor cell expressed αvβ3 without inhibiting host integrins could inhibit lung metastasis, we chose a lung colonization model of human melanoma metastasis in SCID mice. In this model, c7E3 Fab only binds to the human tumor cell expressed integrin but not to the host (mouse) integrin. A single pretreatment of human melanoma HT168M1 cells with c7E3 Fab (2.5 μg/ml) significantly decreased the number and size of tumor colonies in the mouse lung (Fig. 8). These results collectively suggest that blockade of tumor cell αvβ3 can provide antimetastatic benefit by blocking tumor cell-platelet, tumor cell-endothelium, and platelet-endothelium interactions.

Inhibition of bFGF-stimulated Angiogenesis in Nude Rats. Results described above indicate that c7E3 Fab is a potent antimetastatic agent *in vivo* and an antiangiogenic agent *in vitro*. Next, we determined whether endothelial αvβ3 and platelet GPIIb/IIIa integrins were involved in angiogenesis *in vivo*. c7E3 Fab does not cross-react with mouse or rat integrins; however, m7E3 F(ab')$_2$ cross-reacts with rat integrins (26); therefore, we evaluated its antiangiogenic activity *in vivo* using a bFGF-stimulated Matrigel plug angiogenesis model. m7E3 F(ab')$_2$ at 6 mg/kg completely inhibited *ex vivo* rat platelet

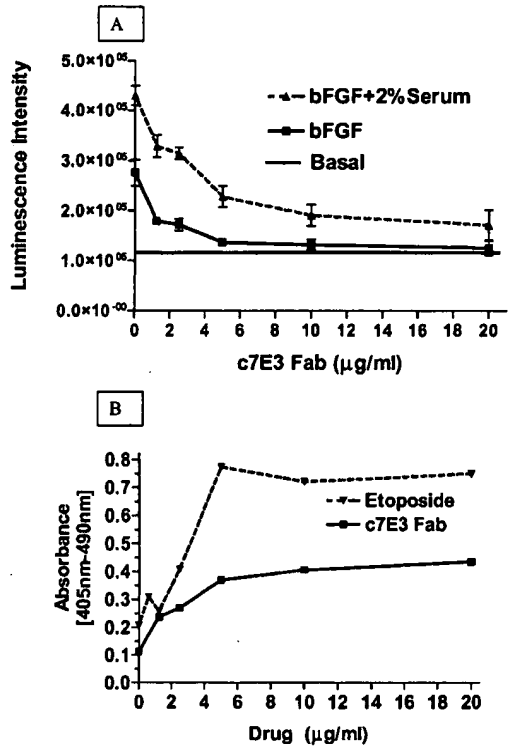

Fig. 6. c7E3 Fab inhibits cell proliferation by promoting apoptosis. Cell proliferation and apoptosis assays were performed as described in "Materials and Methods." *A*, c7E3 Fab inhibits bFGF and bFGF +2% serum stimulated proliferation of human endothelial cells. The data are plotted as luminescence intensity, which is directly proportional to cell number. Basal represents luminescence intensity observed in the absence of bFGF and serum. *B*, c7E3 Fab promotes apoptosis of proliferating endothelial cells. HUVECs were cultured in the presence of bFGF as described in *A* and various concentrations of the positive control (etoposide) or c7E3 Fab. Absorbance on the *Y* axis is directly proportional to extent of apoptosis. Each point represents the mean of triplicate determinations; *bars*, ± SD.

aggregation (26); therefore, this dose of the antibody was used to determine whether m7E3 F(ab')$_2$ could inhibit bFGF-stimulated angiogenesis. The antibody was administered daily for 1 week, Matrigel plugs were removed, and hemoglobin content indicated that m7E3 F(ab')$_2$ significantly inhibited angiogenesis in nude rats (Fig. 9). These data demonstrate that blockade of rat αvβ3 and GPIIb/IIIa can inhibit angiogenesis in rats.

m7E3 F(ab')$_2$ Inhibits Growth of Human Melanoma Tumors in Nude Mice and in Nude Rats. Because c7E3 Fab inhibits human melanoma cell adhesion and invasion *in vitro*, we explored whether m7E3 F(ab')$_2$ could inhibit tumor growth independent of blocking angiogenesis in a human melanoma xenograft model in nude mice. In this model, m7E3 F(ab')$_2$ only blocks human tumor integrins but not mouse integrins. Antibody therapy (10 mg/kg) was initiated 3 days after tumor cell inoculation, and the dosing regimen was three times per week for the duration of the study. Fig. 10 indicates that m7E3 F(ab')$_2$ partially inhibited growth of human melanoma tumors in nude mice (P = 0.0002). These results provide direct evidence that blockade of human melanoma cell-expressed αvβ3 integrin, without inhibiting mouse β3 integrins, can partially inhibit tumor growth *in vivo*.

We hypothesized that combined blockade of both tumor cell-expressed αvβ3 and the host β3 integrins (platelet GPIIb/IIIa and endothelial αvβ3) may result in enhanced inhibition of tumor growth *in vivo*. To test this hypothesis, the same human melanoma A375S2 cells used in the mouse studies were used in a rat model where m7E3 F(ab')$_2$ blocks multiple integrins: platelet GPIIb/IIIa, endothelial αvβ3, and tumor cell αvβ3. This model mimics the clinical situation where the relevant integrins are expressed by the tumor and the host. A dose of 6 mg/kg of m7E3 F(ab')$_2$ was used, because at this concentration the antibody completely inhibited *ex vivo* rat platelet aggregation (26) and inhibited angiogenesis in nude rats (Fig. 9). Two series of experiments were performed in nude rats. In the first experiment, antibody and tumor cells were coinoculated into nude rats, and

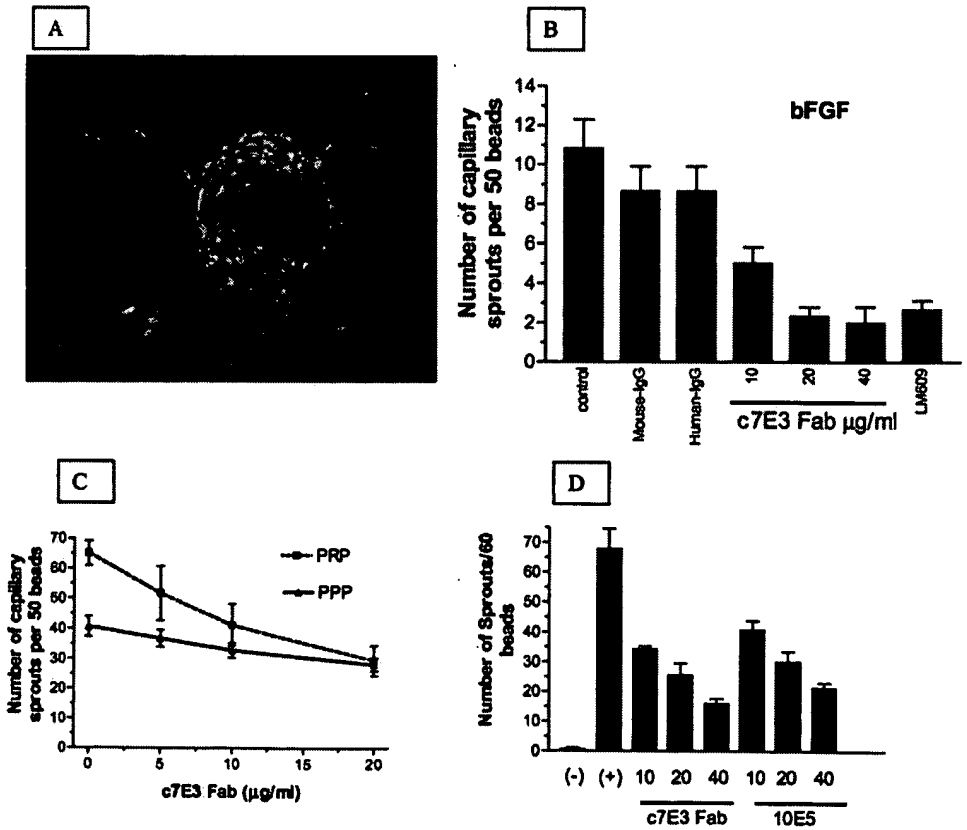

Fig. 7. c7E3 Fab inhibits sprouting of human endothelial cell. *A*, digital photomicrograph of a representative bead coated with HUVECs cultured in a fibrin gel. The number of microvessels sprouting from the bead (total sprouts/50 beads) was quantified as described in "Materials and Methods." *B*, effect of c7E3 Fab on bFGF-stimulated endothelial sprouting. *Control bar* represents sprouting in the absence of antibody. Mouse IgG and human IgG were used at 20 μg/ml as negative control antibodies, and LM609 was used at 20 μg/ml. *C*, dose-dependent inhibition of endothelial sprouting by c7E3 Fab in the presence of PRP and PPP. *D*, dose-dependent inhibition of endothelial cell sprouting by c7E3 Fab and 10E5 in the presence of gel-filtered platelets. −, sprouting in the absence of gel-filtered platelets; +, maximum sprouting in the presence of platelets and mouse IgG (20 μg/ml). Each data point is mean of at least triplicate determinations, and the graphs are representative of two separate experiments; *bars*, ± SD.

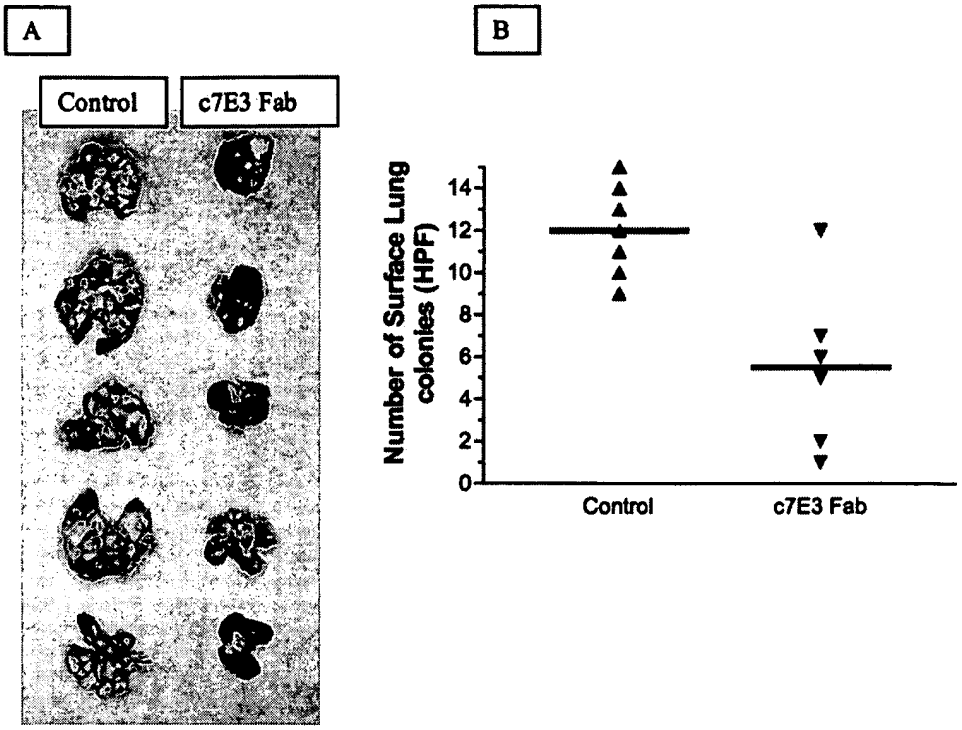

Fig. 8. Effect of c7E3 Fab on lung metastasis of human melanoma cells. Human melanoma HT168M1 cells were incubated with control antibody (2.5 μg/ml) or c7E3 Fab (2.5 μg/ml) for 30 min at room temperature, cells were centrifuged, resuspended, and 1×10^6 cells were tail vein injected into male SCID mice. After 2 months, animals were euthanized, and lungs were removed as described in "Materials and Methods." *A*, photographs of representative lungs treated with control antibody or c7E3 Fab. *B*, the number of surface lung colonies were counted under stereomicroscope. Each data point represents one animal, and the line is the median of the data points. A one-tailed *t* test analysis indicated that c7E3 Fab significantly decreased the number of tumor colonies on the lung surface ($P = 0.0015$) and the weight of tumor-bearing lungs ($P = 0.0463$, data not shown).

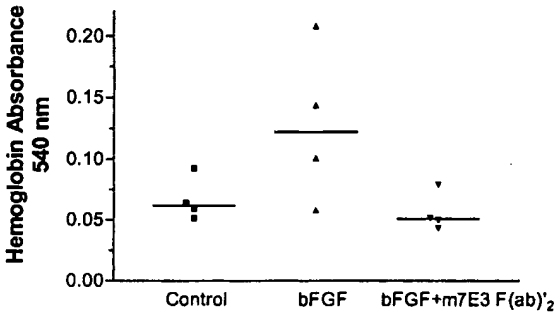

Fig. 9. m7E3 F(ab')$_2$ inhibits bFGF-stimulated angiogenesis in Matrigel plugs in nude rats. The Matrigel plug angiogenesis assay was performed as described in "Materials and Methods." Each point represents hemoglobin content from a Matrigel plug obtained from one animal, and the line represents the median of all values within the group. A one-tailed *t* test analysis indicated that m7E3 F(ab')$_2$ significantly decreased bFGF-mediated angiogenesis ($P = 0.034$).

antibody therapy was administered either 3x/wk or 5x/wk for the duration of the study. Tumor formation and growth were dramatically inhibited in both antibody treated groups (Fig. 11). Animals treated with m7E3 F(ab')$_2$ 3x/wk developed measurable tumors 10 days later compared with the control group, and animals treated with the antibody 5x/wk did not develop tumors throughout the course of the study. Only 50% of the animals in the 3x/wk antibody treatment group developed tumors, and these tumors were significantly smaller compared with the control group ($P < 0.001$; Fig. 11).

To determine whether m7E3 F(ab')$_2$ could inhibit growth of preformed tumors in nude rats, human melanoma A375S2 cells were inoculated into nude rats, tumors were allowed to grow up to ~150 mm^3, and animals were randomized and then treated with m7E3 F(ab')$_2$ (3 mg/kg, daily i.p. administration for the duration of the study) or vehicle control. Assays performed on terminal blood samples demonstrated that m7E3 F(ab')$_2$ inhibited *ex vivo* platelet aggregation and did not cause thrombocytopenia in any of the animals (data not shown; Ref. 26). m7E3 F(ab')$_2$ was administered more frequently in rats compared with the mice, because it has a much shorter circulating half-life in rats. Approximately 150 μg/ml of m7E3 F(ab')$_2$ was detected in the mouse serum the day after the last dose, whereas ~3 μg/ml of circulating antibody was measured in the rat serum the day after the last dose (data not shown), suggesting that the antibody has a shorter circulating half-life in rats compared with mice. Yet, m7E3 F(ab')$_2$ completely prevented growth of preformed tumors in the rat model (Fig. 11) but only had a partial effect in the mouse model (Fig. 10). Collectively, these data provide evidence that combined antitumor and antiangiogenic targeting by m7E3 F(ab')$_2$ is superior than antitumor targeting alone.

DISCUSSION

The salient findings of this study are that platelet GPIIb/IIIa, and endothelial and tumor cell-expressed αvβ3 participate in angiogenesis, tumor growth, and metastasis. Combined blockade of these receptors on three cell types was more effective at inhibiting tumor

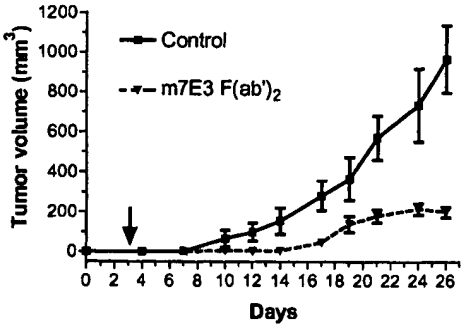

Fig. 10. m7E3 F(ab')$_2$ inhibits growth of human melanoma tumors in nude mice. Human melanoma A375S2 cells (2×10^6) were injected s.c. into female nude mice, and m7E3 F(ab')$_2$ (10 mg/kg 3x/week) or vehicle control therapy was initiated 3 days later as indicated by the *arrow*. Tumor volume measurements were recorded as described in "Materials and Methods." Data points are mean tumor volumes from 10 animals/group; *bars*, ± SD. A one-tailed *t* test analysis indicated that m7E3 F(ab')$_2$ treatment partially inhibited tumor growth on day 26 when compared with control-treated tumors ($P = 0.0002$).

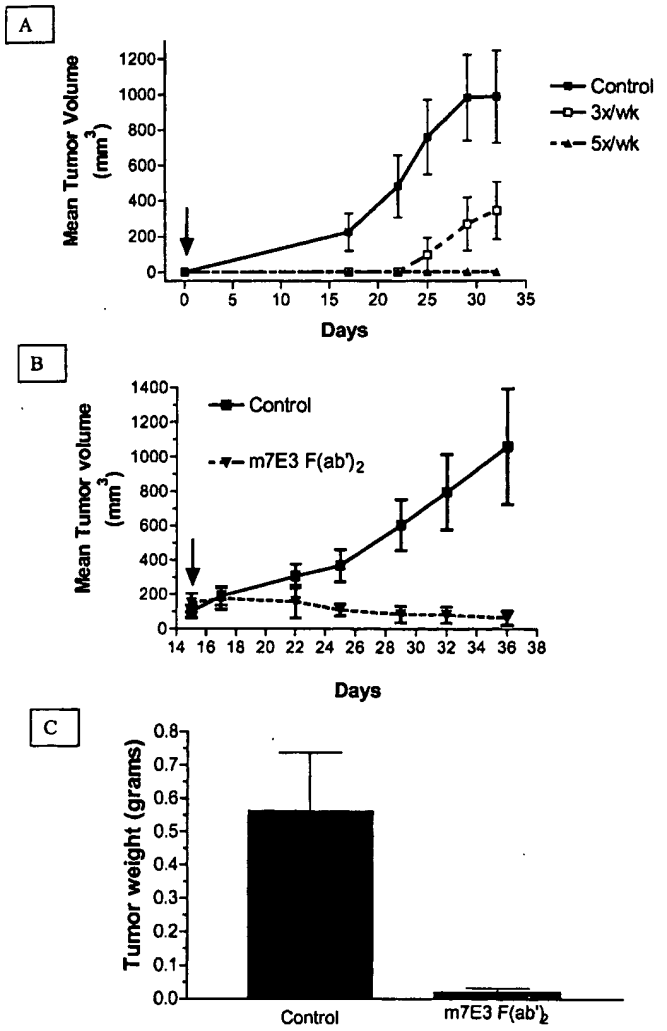

Fig. 11. m7E3 F(ab')$_2$ inhibits growth of human melanoma tumors in nude rats. Human melanoma cells were treated as described in Fig. 10, and they were injected into female nude rats. *Arrow* indicates initiation of therapy. *A*, human melanoma cells were coinjected with m7E3 F(ab')$_2$ or vehicle control, and therapy was initiated on the same day. m7E3 F(ab')$_2$ at a dose of 6 mg/kg or vehicle control was injected either 3x/week or 5x/week. Each data point represents the mean of 6 animals/group; *bars*, ± SD. *B*, human melanoma cells were injected into nude rats, and antibody therapy was initiated 15 days post-tumor cell inoculation after tumors had reached a measurable volume of ~150 mm^3. Antibody or vehicle control was injected from day 15 until day 35 at a dose of 3 mg/kg (daily i.p. injections), and tumor volume measurements were recorded as described in "Materials and Methods." Each data point represents the mean tumor volume from 5 animals in the control and 4 animals in the m7E3 F(ab')$_2$-treated group; *bars*, ± SD. *C*, final weight of tumors excised on day 36 from experiment described in *B*. Bars represent the mean tumor weight from in the control (n = 5) and in the m7E3 F(ab')$_2$ (n = 4)-treated groups; *bars*, ± SD.

growth when compared with blockade of a single integrin receptor. c7E3 Fab, which binds with equivalent affinity to platelet GPIIb/IIIa and αvβ3, inhibited human melanoma and endothelial cell adhesion, migration, invasion, and lung colonization of human melanoma cells in nude mice. In addition, m7E3 F(ab')$_2$ inhibited angiogenesis and growth of human melanoma tumors *in vivo*. Collectively, our results suggest that c7E3 Fab and m7E3 F(ab')$_2$ with their multireceptor activity possess antiangiogenic and antimetastatic properties.

The requirement of platelets in hematogeneous spread of tumor cells was recognized almost 30 years ago and is reviewed in detail elsewhere (1, 31, 32). When metastatic tumor cells are shed into the blood circulation, they rapidly recruit platelets to form tumor cell-platelet aggregates, which results in a transient decrease in circulating platelet count (15, 33). Several preclinical animal models have demonstrated that blockade of platelet GPIIb/IIIa integrin inhibits lung colonization of tumor cells (15, 34). By blocking tumor cell-expressed αvβ3 integrin without inhibiting platelet function, c7E3 Fab, in this study, dramatically inhibited the metastatic ability of human melanoma cells in SCID mice. In this animal model, c7E3 Fab did not cross-react with mouse platelets; therefore, the results demonstrate that human melanoma cell-expressed αvβ3 integrin participates in lung metastasis.

In addition to facilitating metastasis, platelets can also stimulate tumor-induced angiogenesis. Platelet granules contain a variety of angiogenic factors such as VEGF that are rapidly secreted on platelet activation. Previous studies have revealed that an increase in platelet count is an indicator of poor prognosis in patients with lung and ovarian carcinoma (18–20), and platelet-secreted VEGF is inversely correlated with survival of patients with cancer (35). Pinedo *et al.* (12) have postulated that a true antiangiogenic agent must target platelets, but direct evidence to support this hypothesis is lacking. Our data provided novel evidence to support this hypothesis and demonstrate that platelets stimulated endothelial sprouting *in vitro*, and c7E3 Fab inhibited this sprouting. Earlier studies demonstrated that c7E3 Fab inhibited secretion of VEGF from platelets (15, 16); therefore, it is conceivable that VEGF could be contributing to platelet-stimulated endothelial cell sprouting. Platelet-secreted VEGF is probably not the only factor that stimulates angiogenesis, because platelets also contain other growth factors such as TGF-β and thrombin (13) that can stimulate endothelial cell sprouting. c7E3 Fab inhibits platelet-endothelial binding (36) and secretion of platelet granules containing growth factors (13), which may explain why c7E3 Fab completely blocked platelet-stimulated endothelial cell sprouting. This is an important finding, because it demonstrates that not just tumor cells, but host cells can contribute to tumor angiogenesis.

In addition to blocking platelet GPIIb/IIIa, abciximab also inhibits αvβ3 function. Because αvβ3 is an essential receptor for angiogenesis, c7E3 Fab can inhibit endothelial cell proliferation, adhesion, migration, invasion, and induce apoptosis of proliferating cells. Human melanoma cell-expressed αvβ3 participates in cell adhesion, migration, and invasion, and increase in β3 integrin inversely correlates with survival of melanoma patients (9, 10). c7E3 Fab completely inhibited αvβ3-mediated human melanoma cell adhesion, spreading, and invasion. More importantly, m7E3 F(ab')$_2$ has direct antitumor activity *in vivo*. Blockade of human melanoma cell-expressed αvβ3 by m7E3 F(ab')$_2$, without blocking host cell integrin, resulted in a partial inhibition of tumor growth in nude mice. Interestingly, combined blockade of host integrins (platelet GPIIb/IIIa and endothelial αvβ3) and tumor cell-expressed αvβ3 completely prevented tumor formation and growth in nude rats. In this rat xenograft model, which mimics the clinical situation, combined antiangiogenic and antitumor activity of m7E3 F(ab')$_2$ was superior at inhibiting tumor growth when compared with its antitumor activity in the mouse xenograft model.

Tumor growth and angiogenesis involves multiple integrin receptors; therefore, monospecific αvβ3 antagonists may not be effective at inhibiting tumor progression. Agents that block multiple integrin receptors may be more effective at inhibiting tumor growth and angiogenesis. This study provides novel evidence that combined inhibition of αvβ3 and GPIIb/IIIa may be an effective approach to inhibiting tumor growth, angiogenesis, and metastasis.

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Abciximab Suppresses the Rise in Levels of Circulating Inflammatory Markers After Percutaneous Coronary Revascularization

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Background—Previous investigators have shown that systemic markers of inflammation may be increased in patients with acute ischemic syndromes or after percutaneous coronary revascularization and that persistent elevation in these markers is predictive of excess risk of subsequent adverse cardiac events. By virtue of its cross-reactivity with the glycoprotein IIb/IIIa, avβ3, and αMβ2 receptors, abciximab may reduce inflammatory processes.

Methods and Results—Assays for the inflammatory markers C-reactive protein, interleukin-6, and tumor necrosis factor-α were performed on serum samples obtained from 160 patients in a placebo-controlled, randomized trial of abciximab during angioplasty. Eighty patients each had received a placebo or abciximab bolus plus a 12-hour infusion. Serum samples were drawn at baseline (before revascularization), 24 to 48 hours after study drug administration, and 4 weeks after study drug administration. Between baseline and 24 to 48 hours, the increase in C-reactive protein was 32% less in patients receiving abciximab than placebo (P=0.025); the rise in interleukin-6 levels was 76% less in the abciximab group (P<0.001); and the rise in tumor necrosis factor-α levels was 100% less with abciximab therapy (P=0.112). By 4 weeks, most marker levels had returned to baseline, with no significant differences between placebo and abciximab groups.

Conclusions—Systemic markers of inflammation increase in the first 24 to 48 hours after angioplasty, but the magnitude of that rise is diminished by periprocedural abciximab. Some of the long-term clinical benefit derived from this agent may be related to an anti-inflammatory effect. (***Circulation.*** **2001;104:163-167.)**

Key Words: inflammation ■ angioplasty ■ platelets

The contribution of inflammation to the pathogenesis of atherosclerotic heart disease and the complications of acute ischemic syndromes and coronary revascularization has been increasingly recognized over the last several years. Elevated serum levels of acute phase proteins, which are nonspecific systemic markers of inflammation, have been associated with elevated risk for both short-term and long-term ischemic events after successful coronary angioplasty or stenting[1–4] and among patients with unstable angina[5–7] or myocardial infarction.[8]

Pharmacological agents directed against the integrin glycoprotein (GP) IIb/IIIa receptor potently inhibit platelet aggregation and thrombus formation at the injured coronary plaque.[9] These agents reduce the risk of acute ischemic events by up to 50% among patients undergoing angioplasty, stenting, or atherectomy.[10] In addition to the antithrombotic effect, however, blockade of the GP IIb/IIIa receptor may inhibit the interaction between platelets and leukocytes, and hence limit the inflammatory response to coronary intervention. Moreover, cross-reactivity of one of the GP IIb/IIIa inhibitors, abciximab (ReoPro, Centocor), with other integrin receptors, including αMβ2 (Mac-1), may have other anti-inflammatory effects.[11,12]

To assess whether abciximab influences the inflammatory process in patients undergoing percutaneous coronary revascularization, we measured periprocedural changes in circulating levels of systemic inflammatory markers in patients who were enrolled in the Evaluation of c7E3 for Prevention of Ischemic Complications (EPIC) randomized, placebo-controlled trial of abciximab during balloon angioplasty.

Methods

Patient Population and EPIC Trial Protocol

The patient population described in this study is a subset of 160 patients enrolled in the EPIC trial.[13] In brief, 2099 patients undergoing high-risk coronary balloon angioplasty or directional atherec-

Received January 25, 2001; revision received April 17, 2001; accepted April 17, 2001.

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Drs Lincoff, Kereiakes, and Topol have received research support from Centocor, Inc; Drs Mascelli, Deckelbaum, Barnathan, Patel, Frederick, and Nakada are employees of Centocor, Inc.

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TABLE 1. Patient Baseline Characteristics

	Inflammatory Substudy Patients			Overall EPIC Trial*
	Placebo	Abciximab	Total	
n	80	80	160	1404
Age, y	60.4±8.6	60.3±9.2	60.4±8.9	60.2±10.6
Female sex, %	26.3	18.8	22.5	27.9
White, %	93.8	90.0	91.9	92.6
Diabetes mellitus, %	27.5	30.0	28.8	24.4
Hypertension, %	53.8	51.3	52.5	54.0
Elevated cholesterol, %	60.3	57.1	58.7	56.2
History of smoking, %	21.3	21.3	21.3	31.6
Peripheral vascular disease, %	8.9	10.0	9.4	8.8
Prior MI, %	12.5	11.4	11.9	13.4
Prior coronary angioplasty, %	26.3	35.0	30.6	23.1
Prior coronary surgery, %	18.8	20.0	19.4	15.5
Acute/recent MI†	0	0	0	28.6
Unstable angina	18.8	13.8	16.3	23.7

Continuous variables are expressed as mean±SD. Dichotomous variables are expressed as percent of total. MI indicates myocardial infarction.

*Excluding patients in the abciximab bolus–only group.

†Acute/recent MI defined as within the prior 7 days.

tomy were enrolled at 56 clinical sites throughout the United States between November 1991 and November 1992. Criteria constituting high-risk status included acute or recent myocardial infarction, unstable angina, complex target lesion angiographic morphology, or a moderately complex target lesion in association with age >65 years, female sex, or diabetes mellitus. The protocol was approved by the Institutional Review Board at each clinical site, and all patients gave informed consent.

All patients were treated with aspirin and sufficient heparin to achieve an activated clotting time >300 to 350 seconds before and throughout the coronary intervention. Patients were randomized in a double-blind fashion to 1 of 3 intravenous treatment regimens: placebo, abciximab 0.25 mg/kg bolus, or abciximab 0.25 mg/kg bolus followed by a 10 μg/min infusion for 12 hours. Coronary angioplasty or directional atherectomy were performed according to conventional techniques.

Inflammatory Markers Substudy Protocol

Blood samples were scheduled to be drawn in all patients in the EPIC trial at baseline (preintervention), hospital discharge, and 2, 4, and 12 weeks after study drug administration for measurement of the presence of human anti-chimeric antibodies. Sufficient serum was available for each patient, beyond that required for antibody testing, for aliquots to be preserved for future analyses. The patients included in this substudy were the first consecutive 160 patients enrolled (80 each randomized to placebo or abciximab bolus plus infusion) who had not sustained a recent (within 7 days) myocardial infarction and who had sufficient serum samples available at baseline, 24 to 48 hours after drug administration, and 4 weeks (25 to 31 days) after administration. Patients randomized to the abciximab bolus-only arm of the trial were not included in this inflammatory markers substudy because of the lack of clinical efficacy of that dosage regimen.

Blood was drawn into red top Vacutainer tubes and allowed to clot at room temperature before centrifugation to separate the serum. Serum was collected and aliquoted into 2-mL Sarstedt microtubes, frozen, and shipped from the clinical sites to Centocor on dry ice. Samples were maintained at Centocor at −70°C until the inflammatory assays were performed. Serum was analyzed for levels of high-sensitivity C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) at the Mayo Central Laboratory for Clinical Trials. CRP was measured on the Hitachi 912 chemistry analyzer by a latex particle–enhanced immunoturbidimetric assay (Kamiya Biomedical Corp). Intra-assay coefficients of variation were 8.8%, 1.1%, and 0.4% at CRP concentrations of 0.028, 0.20, and 1.15 mg/dL, respectively. IL-6 was measured by a 2-site sequential chemiluminescent isometric assay on the Immulite automated immunoassay system (Diagnostic Products). Intra-assay coefficients of variation were 7.5%, 7.3%, and 4.2% at IL-6 concentrations of 12.4, 20, and 108 pg/mL, respectively. TNF-α was measured by a solid phase, 2-site chemiluminescent immunometric assay on the Immulite automated assay system. Intra-assay coefficients of variation were 12.1%, 7.0%, and 4.7% at TNF-α concentrations of 6.8, 8.5, and 282 pg/mL, respectively.

Statistical Methods

Continuous variables were summarized by means, medians, SDs, and interquartile ranges. Changes in levels of serum inflammatory markers at 24 to 48 hours and at 4 weeks were assessed relative to baseline (before study drug administration). Differences between placebo and abciximab treatment groups with respect to changes in serum markers were compared using the nonparametric Wilcoxon 2-sample test, because the data were not normally distributed. A sample size of 160 was calculated to produce an 80% power to detect a 30% difference between treatment groups with regard to the rise in inflammatory markers with a significance level of 0.05.

Results

Baseline Characteristics and Clinical Outcome

Baseline characteristics were balanced between the placebo and abciximab groups in the inflammatory markers substudy (Table 1). Patients within the substudy were largely representative of the overall EPIC trial population, although a smaller proportion of substudy patients had an acute ischemic syndrome, because of the exclusion of patients with recent myocardial infarction. The composite end point of death, myocardial infarction, or urgent revascularization by 30 days after randomization occurred in 7 patients in the placebo group (8.8%) and 1 patient in the abciximab bolus plus

TABLE 2. Inflammatory Marker Levels

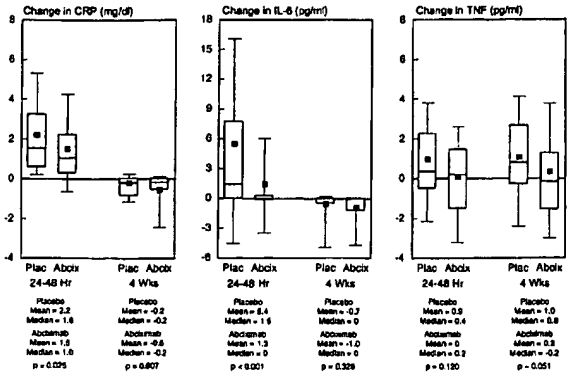
	Placebo (n=80)	Abciximab (n=80)
CRP, mg/dL		
Baseline		
Mean±SD	0.9±0.9	1.2±1.7
Median (IQR)	0.7 (0.3, 1.3)	0.5 (0.2, 1.3)
24–48 Hours		
Mean±SD	3.1±2.9	2.7±2.3
Median (IQR)	2.2 (1.2, 4.2)	2.3 (1.0, 3.7)
4 Weeks		
Mean±SD	0.7±1.4	0.6±1.0
Median (IQR)	0.3 (0.1, 0.7)	0.3 (0.1, 0.7)
IL-6, pg/mL		
Baseline		
Mean±SD	6.2±2.3	6.5±3.2
Median (IQR)	5.0 (5.0, 6.0)	5.0 (5.0, 6.5)
24–48 Hours		
Mean±SD	11.6±11.9	7.9±6.8
Median (IQR)	7.4 (5.0, 13.4)	5.0 (5.0, 7.7)
4 Weeks		
Mean±SD	5.5±1.9	5.5±4.0
Median (IQR)	5.0 (5.0, 5.0)	5.0 (5.0, 5.0)
TNF-α, pg/mL		
Baseline		
Mean±SD	6.0±2.2	7.1±3.1
Median (IQR)	5.7 (4.0, 7.2)	6.9 (5.1, 8.1)
24–48 Hours		
Mean±SD	6.9±3.7	7.2±3.2
Median (IQR)	6.3 (4.3, 8.1)	6.8 (5.0, 8.4)
4 Weeks		
Mean±SD	7.0±3.0	7.4±3.6
Median (IQR)	6.1 (4.6, 8.3)	6.6 (5.2, 8.5)

IQR indicates interquartile range. Lower limits of assay sensitivity were as follows: CRP, 0.02 mg/dL; IL-6, 5 pg/mL; TNF-α, 4 pg/mL.

infusion group (1.3%; $P=0.03$), which reflects the treatment effect of abciximab observed in the overall trial population.[13]

Inflammatory Markers

Levels of the 3 inflammatory markers before (baseline), 24 to 48 hours after, and 4 weeks after study drug administration are presented in Table 2. Changes in the levels of these markers relative to baseline are presented in the Figure. The increase in CRP level between baseline and 24 to 48 hours was 32% less in patients receiving abciximab compared with those receiving placebo ($P=0.025$), with no significant differences between treatment groups in the changes in levels out to 4 weeks. The observed rise in IL-6 levels at 24 to 48 hours was 76% less in the abciximab group than in the placebo arm ($P<0.001$), with no differences in the changes in levels at 4 weeks. The rise in TNF-α levels was 70% to 100% less in the abciximab group at both 24 to 48 hours ($P=0.112$) and 4 weeks ($P=0.051$).



Box-and-whisker plot of changes in levels of CRP (left), IL-6 (center), and TNF-α (right) by 24 to 48 hours and 4 weeks relative to baseline (before study drug administration). The box spans the interquartile range (25th to 75th percentiles), and the line within the box denotes the median. Whiskers extend from the 10th to 90th percentiles. The square represents the mean. Abcix indicates abciximab bolus plus infusion; Plac, placebo.

To assess whether the observed effect of abciximab on reducing the rise in inflammatory markers by 24 to 48 hours was due to the prevention of ischemic events, the analysis was repeated after excluding the 4 patients in the placebo group and the 1 patient in the abciximab group who had experienced an ischemic end point (myocardial infarction or urgent repeat revascularization) within the first 48 hours after study drug administration. Changes in the levels of the 3 markers by 24 to 48 hours in this subgroup were nearly identical to those observed in the overall cohort of patients. The mean rise in CRP was 2.2±2.4 mg/dL versus 1.5±2.2 mg/dL in the placebo and abciximab groups, respectively ($P=0.028$); mean rise in IL-6 was 5.4±12.1 pg/mL and 1.4±7.2 pg/dL, respectively ($P=0.001$); and the mean rise in TNF-α was 0.9±3.3 pg/mL and 0±2.4 pg/mL, respectively ($P=0.130$).

Discussion

Blockade of the platelet GP IIb/IIIa receptor with abciximab markedly decreases the risk of ischemic complications from percutaneous coronary revascularization, an effect which has traditionally been attributed to inhibition of platelet aggregation and thrombus formation. This current study demonstrates for the first time that abciximab also suppresses the periprocedural rise in markers of systemic inflammation. Among a subgroup of 160 patients in the placebo-controlled EPIC trial, levels of CRP, IL-6, and TNF-α increased over the 24 to 48 hours after high-risk balloon angioplasty or atherectomy. Treatment with abciximab, however, was associated with reductions of 30% to 100% in the magnitude of rise in these markers. The influence of abciximab on inflammatory markers seemed to occur independently of the inhibition of ischemic events by this agent. These findings suggest that some of the immediate or long-term benefit of abciximab in the setting of coronary intervention may be related to the suppression of inflammation.

Inflammatory responses have been implicated as important causative factors across the spectrum of acute and chronic

phases of atherosclerotic vascular disease.[14] Endothelial injury induces the expression of intercellular adhesion molecules and the release of chemoattractant compounds that mediate the recruitment, attachment, and migration of leukocytes into the arterial wall.[15] Infiltrating inflammatory cells enhance oxidation and uptake of low-density lipoproteins and produce cytokines, mitogens, and reactive oxygen species, stimulating smooth muscle cell migration and proliferation and contributing to ongoing endothelial injury.[16] These processes lead to the formation of the fatty streak and the mature atherosclerotic plaque. Inflammation also seems to play a role in the development of acute ischemic syndromes. Lymphocytes and monocytes accumulate at the edges of the fibrous cap, producing cytokines and matrix metalloproteinases, which enhance collagen and elastin degradation.[14,17] The resultant evolution of the vulnerable plaque provides the substrate for plaque rupture, vascular thrombosis, and unstable angina or myocardial infarction. Inflammatory processes further perpetuate the thrombotic response to plaque disruption. Binding activated platelets to leukocytes facilitates thrombosis by activating factor X and providing sites for assembly of the prothrombinase complex. Platelet-leukocyte aggregates also enhance the local and systemic inflammatory state by releasing cytokines.

Markers of inflammation are consistently found to be prognostic for the prevalence of atherosclerosis, clinical manifestations of coronary artery disease, and increased risk for complications of acute ischemic syndromes or revascularization procedures. Prospective and cross-sectional studies have documented associations between levels of CRP in apparently healthy individuals and the occurrence of myocardial infarction, stroke, or cardiovascular mortality.[18] Among patients with acute ischemic syndromes, elevated circulating concentrations of CRP, IL-1 receptor antagonist, or IL-6 are predictive of recurrent ischemia, myocardial infarction, and long-term mortality.[5–8] Inflammatory markers have also been shown to increase in the period immediately after percutaneous revascularization procedures in patients with or without unstable myocardial ischemia, and the magnitude of rise in these markers has been correlated with subsequent myocardial infarction and restenosis.[1–4,19]

The efficacy of percutaneous revascularization is considerably improved by the administration of GP IIb/IIIa receptor antagonists. In randomized placebo-controlled trials, the risk of death, myocardial infarction, or emergency repeat revascularization within 30 days after coronary intervention was reduced by ≈40% to 60% with abciximab and by 15% to 35% with eptifibatide (Integrilin, COR Therapeutics) or tirofiban (Aggrastat, Merck).[10] With abciximab, clinical benefit was particularly marked among patients revascularized in the setting of unstable angina.[20] Moreover, this agent has been associated with a long-term decrease in mortality, an effect that cannot be entirely attributed to the suppression of acute periprocedural ischemic events.[21] Mortality reduction has not been observed to date with eptifibatide or tirofiban. Apparent heterogeneity in the magnitude of treatment effect observed in these trials between the antibody fragment and the reversible small molecule inhibitors may reflect differences in the intensity and duration of receptor blockade, inadequate dosing, variations in trial design, or statistical chance. However, differences in receptor specificity among the agents may also be important. Eptifibatide and tirofiban inhibit only GP IIb/IIIa, but abciximab also binds to the $\alpha v\beta 3$ (vitronectin) receptor on endothelial, smooth muscle, and inflammatory cells[11] and to an activated conformation of the $\alpha M\beta 2$ receptor on leukocytes.[12]

The cross-reactivity of abciximab raises the possibility that clinical benefit derived from this therapy may not be exclusively due to its antithrombotic effect, but may also be related to the suppression of inflammatory pathways involving platelets, white blood cells, and the vascular endothelium. Leukocytes initially adhere to endothelial cells via P-selectin, but firmer attachment is mediated by $\alpha M\beta 2$ binding, either directly[22] or through fibrinogen bridging to intracellular adhesion molecule-1 on endothelial cells.[23] Fibrinogen bridging of $\alpha M\beta 2$ and intracellular adhesion molecule-1 also enhances leukocyte migration across the endothelium.[24] One of the 2 complementary mechanisms of formation of platelet-leukocyte aggregates involves fibrinogen bridging of platelet GP IIb/IIIa to leukocyte $\alpha M\beta 2$.[25] Abciximab may not only inhibit these inflammatory processes directly by blocking GP IIb/IIIa and $\alpha M\beta 2$, but it also seems to reduce leukocyte surface expression of $\alpha M\beta 2$.[26] Issues of cross-reactivity with other receptors aside, it must be emphasized that GP IIb/IIIa receptor blockade per se is sufficient to reduce platelet attachment to monocytes and endothelial cells, thus exerting an anti-inflammatory effect. Thus, the relative efficacy of the selective versus nonselective GP IIb/IIIa antagonists in reducing periprocedural inflammation can be assessed only by direct comparative studies.

In this current study, we evaluated the effect of abciximab on the rise in levels of CRP, IL-6, and TNF-α after percutaneous revascularization. TNF-α and IL-6 are cytokines that mediate humoral and cellular inflammatory processes in response to infection, inflammation, and tissue injury. IL-6 is expressed in a number of cell types, including those within atherosclerotic plaque, and its production seems to be controlled in part by IL-1β and TNF-α.[6,19] CRP is an acute-phase reactant produced by the liver under the influence of inflammatory cytokines, principally IL-6.[19] In addition to acting as a marker of a systemic inflammatory state, CRP may also have a direct pro-inflammatory effect,[27] and it may influence thrombosis and inflammation through complement activation.[28] Consistent with previous investigations,[1–3,19] we observed an increase in serum levels of these markers, particularly CRP and IL-6, over the first 24 to 48 hours after coronary intervention. The observed suppressive effect of abciximab on the rise in IL-6 was somewhat greater than for CRP, perhaps reflecting the greater stability of CRP in the circulation (half-life of 19 hours versus 4 hours for IL-6).[19] It does not seem that myocardial necrosis per se was the source of cytokine elevation in this study; patients with myocardial infarction within the prior 7 days had been excluded from consideration, and results were unchanged when the few patients experiencing postprocedural ischemic events were removed from the analysis. For the same reasons, the diminution of the postprocedural rise in inflammatory marker levels by abciximab seems to have occurred independently of

the reduction in ischemic complications. It is possible, however, that suppression of periprocedural ischemic events below the threshold of clinically detectable myocardial necrosis may have accounted for some of the effect of abciximab on inflammatory responses.

Given the relatively small number of ischemic end points that occurred in this study of 160 patients, it was not possible to detect a correlation between levels of inflammatory markers and subsequent adverse clinical events. Therefore, a cause-and-effect relationship could not be established between suppression of the rise in these markers by abciximab and the known benefits of this agent. Nevertheless, the growing body of evidence linking systemic inflammation to unfavorable short- and long-term outcome in cardiovascular disease states suggests that an anti-inflammatory effect of abciximab may have salutary clinical consequences. These data thus have implications for possible differential efficacy among various GP IIb/IIIa inhibitors and are supportive of a potential role for modifiers of the inflammatory process in reducing ischemic events in patients undergoing percutaneous coronary revascularization.

Acknowledgment

Supported by Centocor, Inc, Malvern, Pa.

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Circulation

(*Circulation.* 2002;105:e74.)

Abciximab Suppresses the Rise in Levels of Circulating Inflammatory Markers After Percutaneous Coronary Revascularization

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To the Editor:

We read with interest the article by Lincoff et al.[1] Abciximab is thought to be superior to other glycoprotein IIb/IIIa inhibitors in the context of percutaneous coronary intervention (PCI) for acute coronary syndromes (ACS). The study by Lincoff et al[1] highlights a potential novel mechanism by which this may occur—namely, inhibiting leukocyte $\alpha_{M\beta 2}$ receptors. Patients with elevated C-reactive protein (CRP) have a higher proportion of activated monocytes and a greater capacity to generate IL-6 in the context of ACS.[2] Because there was no baseline difference in CRP and IL-6 levels between placebo and abciximab treatment groups, the proportion of activated monocytes was similar in the 2 groups. The process of PCI, therefore, generates an inflammatory response that is reduced by abciximab.

An alternative explanation for the results is the inflammatory response to myocardial necrosis. Even uncomplicated elective PCI procedures are accompanied by a rise in cardiac troponins.[3] Myocardial damage results in increased release of IL-6 and hence CRP. Abciximab reduces thrombosis in PCI, which may reduce distal embolization and thus the degree of myocardial damage.[4] Abciximab may reduce the inflammatory response seen in PCI by reducing myocardial damage rather than by having a direct antiinflammatory action. Procedures such as atherectomy

are associated with an increased risk of distal embolization and may be a further confounding factor.

Could the authors clarify whether there was a difference in the 24-hour troponin levels between the placebo and abciximab treatment groups and whether the proportion of patients undergoing atherectomy was similar? The results seen can only be attributed to the inhibition of the $\alpha_{M\beta 2}$ receptor if no differences in troponin levels between the 2 groups are seen.

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Response

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We appreciate the comments on our article[1] by Dr Ray, who makes the very relevant point that part of the antiinflammatory effect of abciximab may have been mediated by a reduction of myocardial necrosis. In our paper, we reported that the findings of the study were unchanged if patients suffering postprocedural myocardial infarction were excluded. However, inflammatory marker levels may have also been influenced by necrosis below the threshold of detection with

creatine kinase-MB. This limitation was acknowledged in the paper, where we noted that "... suppression of periprocedural ischemic events below the threshold of clinically detectable myocardial necrosis may have accounted for some of the effect of abciximab on inflammatory responses." Unfortunately, although troponin is certainly a more sensitive indicator of myocardial damage, these assays were not available and thus were not performed from 1992 through 1993 when the Evaluation of c7E3 for Prevention of Ischemic complications (EPIC) trial was conducted.

Directional atherectomy has certainly been shown in several studies to increase the incidence of periprocedural myocardial infarction. Among the 160 patients in our EPIC substudy, 11 of 80 (14%) and 14 of 80 (18%) underwent atherectomy in the placebo and abciximab groups, respectively. When these patients were excluded from consideration, the principal findings of the study were unchanged; the rise in levels of CRP, IL-6, and TNF-α were substantially lower over the 24 to 48 hours after coronary intervention among patients treated with abciximab compared with placebo. Thus, although an effect mediated by suppression of periprocedural myocardial infarction cannot be excluded, the available evidence from this trial suggests a predominantly direct antiinflammatory effect of abciximab.

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Supplement to
AHJ
American Heart Journal

146, Volume Kereiakes, Number

Inflammation as a therapeutic target: A unique role for abciximab

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Vascular inflammation is a central pathogenic mechanism for both acute coronary syndromes and the vascular response to injury after percutaneous coronary intervention. The magnitude of vascular inflammation has been correlated with adverse late clinical outcomes (death, myocardial infarction, recurrent ischemia, restenosis). Vascular inflammation is also increased in patients with diabetes mellitus. Many adjunctive pharmacotherapies used in the treatment of acute coronary syndromes or during percutaneous coronary intervention have anti-inflammatory effects, which are distinct from their perceived primary mechanism of action. Data in support of the anti-inflammatory effects of abciximab are presented and the role that these effects may play in modulating atherosclerotic plaque stability and late clinical outcomes is discussed. Vascular inflammation represents the "final common pathway" for many disease processes and thus represents the "ultimate therapeutic target" for pharmacologic intervention. (Am Heart J 2003;146:S1-4.)

Vascular inflammation plays an integral role in the pathogenesis of coronary disease. High-sensitivity C-reactive protein (hs-CRP), a nonspecific marker of inflammation and an acute phase reactant, is a powerful predictor of cardiovascular events including stroke, coronary heart disease, peripheral vascular disease, and sudden cardiac death.[1] The prevalence of elevation in CRP depends on the clinical syndrome[2]: <20% of patients with stable angina have elevated CRP, >65% of patients with unstable angina (UA), ~50% of those with myocardial infarction (MI) without UA, and >90% of those patients with UA culminating in acute MI.[3] Patients with acute coronary syndromes (ACS) have an active inflammatory process, which is reflected by the presence of systemic markers.[4] These markers include interleukin 6 (IL-6), an inflammatory cytokine produced by the monocyte-macrophage complex and vascular smooth muscle cells which travels to the liver where it is a precursor for and promotes the formation of CRP.

Both IL-6 and CRP rise together in patients with UA compared to patients with stable angina and reach highest levels in UA patients who experience a major adverse coronary event (MACE).[4] Thus, both the presence and magnitude of elevation in inflammatory markers carries prognostic importance for patients with ACS. Patients with non-ST-elevation ACS who have increased levels of CRP and who undergo angioplasty have an increased incidence of death or MI at 6 months follow-up compared with those patients who have a normal CRP.[5] In fact, elevation of CRP before early revascularization for non-ST-elevation ACS predicts mortality to 5 years.[6] Those patients with the highest CRP levels have the highest mortality in follow-up. Furthermore, patients with plaque inflammation, as reflected by elevated hs-CRP levels before percutaneous coronary intervention (PCI), have an increased incidence of periprocedural MI, restenosis requiring revascularization, and mortality in follow-up.[5]

It is now appreciated that elevation of CRP is correlated with neutrophil activation, which in turn reflects a generalized inflammatory arteritis.[7] As demonstrated by Buffon et al,[7] patients with unstable angina who have either a left coronary "culprit" lesion or a right coronary "culprit" lesion will have a myeloperoxidase

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0002-8703/2003/$30.00 + 0
doi:10.1016/j.ahj.2003.09.003

difference between the aorta and the great cardiac vein that signifies the presence of white cell (neutrophil) activation. As the great cardiac vein subserves the left anterior descending coronary artery distribution, the presence of white cell activation in a "nonculprit artery" distribution for those patients having a right coronary target lesion is evident. Further explanation for this observation can be derived from a recent multivessel intravascular ultrasound (IVUS) study in patients who presented with ACS.[8] In this study, patients with ACS had IVUS of all major epicardial vessels. Interestingly, 80% had at least 1 extra plaque rupture distinct and separate from the "culprit" plaque rupture that led to their hospital admission. In 71% of patients, this "nonculprit" plaque rupture was present in a separate coronary artery.[8] Thus, these patients clearly had a multilesion, multivessel, and multicentric inflammatory process for which the underlying pathogenesis appears to be macrophage infiltration in plaque.[9] The degree of macrophage infiltration in plaque parallels and correlates with coronary disease activity. In addition, patients who have plaque inflammation as reflected by increased CRP levels will have an increased incidence of symptomatic restenosis (restenosis requiring a revascularization procedure). The predilection for restenosis has been correlated with the degree of preprocedural macrophage infiltration of plaque.[10]

Yet another inflammatory cytokine, IL-18, has been shown to predict death in patients with both stable and unstable angina even when adjustments for other known risk factors (including CRP, IL-6, and fibrinogen) are made.[11] These observations further attest to the central role of inflammation in the pathogenesis and prognosis of the atherosclerotic coronary disease process.

PCI, particularly coronary stent deployment, also provokes an inflammatory response, which is proportional to the degree of focal medial injury.[12] Lymphocyte/macrophage infiltration is prevalent both early and late in the sequence of thrombus formation, inflammation, and neointimal proliferation that follows stent deployment.[13] Indeed, neointimal macrophage infiltration is correlated with subsequent in-stent restenosis.[12] Animal studies have shown that when macrophages are depleted by the administration of liposomal clodronate (vs control), the macrophage-depleted animals have little or no scar tissue formation after balloon angioplasty, while controls have an abundance.

The inflammatory response to stenting in man, as reflected by CRP levels, has been profiled by Gaspardone.[14] CRP levels are most elevated at 48 to 72 hours after stent deployment. Furthermore, a CRP level >0.5 mg/dL at 72 hours post-stent deployment is associated with an increased incidence of adverse events (death, MI, or recurrent angina) to 1 year follow-up. Recent data suggest that treatment of this exaggerated inflammatory response to stenting with an anti-inflammatory agent (prednisone) may improve both clinical and angiographic late outcomes (vs placebo in randomized comparison).[15]

Patients who have had plaque stabilization with statin therapy before PCI have a lower incidence of both periprocedural MI and inhospital mortality. Statins and angiotensin converting enzyme (ACE) inhibitors have anti-inflammatory and antithrombotic effects and are associated with a reduction in the incidence of in-stent restenosis and the requirement for revascularization.[16,17] The antithrombotic effect of these agents is, in part, mediated by suppression of tissue factor expression by macrophages and vascular endothelial cells.[18,19] Tissue factor expression has been correlated with macrophage density in plaque from patients with UA.[10]

CRP is not only a marker, but is also a mediator of inflammation. CRP has proinflammatory effects[20,21] (by promoting intracellular adhesion molecule [ICAM], vascular cell adhesion molecule [VCAM], and monocyte chemoattractant protein-1 [MCP-1] expression), proatherogenic effects[22] (by promoting low-density lipoprotein uptake by macrophages), and prothrombotic effects[23-25] (by inhibiting release of tissue factor pathway inhibitor as well as promoting both tissue factor and plasminogen activator inhibitor-1 expression). CRP also attenuates nitric oxide production and suppresses endothelial nitric oxide synthase (eNOS) activity.[26,27] (Nitric oxide has potent anti-inflammatory activity.)

A potent direct and sustained anti-inflammatory effect of abciximab after PCI has been demonstrated.[28] The durability of abciximab's anti-inflammatory effects may, in part, be due to the pharmacodynamic profile of this agent, which exhibits "redistribution" across cellular receptors.[29] Indeed, as reflected by GP IIb/IIIa receptor occupancy, abciximab lingers in the circulation for >2 weeks after bolus administration. Abciximab also binds the vitronectin ($\alpha_v\beta_3$) receptor with the same affinity as the glycoprotein (GP) IIb/IIIa receptor. Inhibition of the vitronectin receptor inhibits leukocyte adherence and leukocyte transmigration across endothelial cells.[30,31] Abciximab also binds to the CD11b/18 (Mac-1) receptor, which plays an active role in modulating white cell adhesion, white cell-platelet interactions, and the white cell inflammatory response to PCI vessel injury.[32]

Abciximab has also been suggested to have a direct plaque stabilizing effect that may be mediated by the Mac-1 receptor. This receptor also modulates the process of monocyte-induced smooth muscle death. When vascular smooth muscle cells are incubated in cell culture with monocytes and monocyte colony stimulating factor (MCSF), marked killing (apoptosis) of the smooth muscle cells was observed.[33] Abciximab, in

physiologic concentrations, was associated with a reduction in smooth muscle cell death (protective effect) similar to that provided by CD-18, a human monoclonal antibody specific to the Mac-1 receptor. The addition of eptifibatide or tirofiban, which provide "pure" GP IIb/IIIa receptor blocking effects (no Mac-1 receptor affinity), provided no benefit to preserve vascular smooth muscle cells in this model. As both macrophage density in plaque[9] and serum MCSF levels are increased in vitro for patients with ACS,[34] this model for modulation of vascular smooth muscle cell death appears to be consistent with pathology studies previously reported.[35,36] Indeed, postmortem histologic studies have suggested that plaques that rupture and cause sudden death or MI are rich in macrophage content and depleted in vascular smooth muscle cell content.[35,36] These observations provide a framework by which abciximab may improve late clinical outcomes (including survival), which is distinct from periprocedural GP IIb/IIIa receptor platelet inhibition.

Diabetes is an active inflammatory process that is marked by increased density of macrophage infiltration of atherosclerotic plaque.[37] It has been suggested that it is the degree of inflammation (as reflected by the level of hs-CRP) that determines the prognosis in diabetes more accurately than the diabetic state per se.[38] A pooled analysis of 4 major placebo-controlled, randomized trials of platelet GP IIb/IIIa inhibitors given to patients with non-ST-elevation ACS showed that patients with diabetes have an increased mortality to 30 days postenrollment when compared with patients without diabetes.[39] Platelet GP IIb/IIIa inhibitor therapy has been associated with a reduction in mortality for patients with diabetes with ACS according to a recent meta-analysis of multiple placebo-controlled randomized trials.[40]

In this analysis, only 1 study demonstrated a statistically significant reduction in mortality in patients with diabetes treated with a GP IIb/IIIa inhibitor—in this case in patients receiving abciximab.[40] This observation is consistent with results from the Evaluation of IIb/IIIa Platelet Inhibitor for STENTing (EPISTENT) trial, in which abciximab (vs placebo) was associated with a >50% reduction in death, MI, and target vessel revascularization (TVR) in patients with diabetes at 6 months, and a 40% reduction in TVR at 1 year.[41] These benefits of abciximab in patients with diabetes (inflammatory state) may, at least in part, be a reflection of its potent and sustained anti-inflammatory effects. The anti-inflammatory effects of abciximab may also play a role in mediating the late clinical benefit associated with abciximab treatment, as demonstrated in a recent pooled analysis of the Evaluation of c7E3 for the Prevention of Ischemic Complications (EPIC), Evaluation of PTCA to Improve Long-term Outcome by c7E3 GP IIb/IIIa Receptor Blockade (EPILOG), and EPISTENT trials—in which survival at 3 years post-PCI was significantly increased in patients who received abciximab (vs placebo, 22% relative reduction in mortality, $P = .03$)[42]—and the completed 7-year follow-up of the EPIC trial, in which patients with ACS (inflammatory state) who received abciximab had an even greater reduction (32%) in late mortality.[42]

In summary, the degree of vascular inflammation determines coronary disease activity and the response to vessel injury (PCI). ACS and diabetes are inflammatory processes. Levels of inflammatory cytokines and markers of inflammation (especially hs-CRP) reflect the status of vascular inflammation. GP IIb/IIIa inhibition with abciximab provides anti-inflammatory effects that appear to be separate and distinct from platelet inhibition and may contribute to the long term clinical benefit derived from this agent.

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